Monitoring the presence and effects of marine litter in Mediterranean MPAs: the Plastic Busters MPAs protocols

PREPARED BY

THE INTERREG MED PLASTIC BUSTERS MPAs PROJECT

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Document Information

This document (Deliverable 5.2.1) is a compilation of all the protocols that should be applied in order to elaborate a comprehensive diagnosis of the presence and effects of marine litter in Mediterranean MPAs.

Approvals

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1. Introduction

1.1 Marine litter a lurking threat in Mediterranean MPAs

The Mediterranean Sea is one of the areas most affected by marine litter worldwide. Marine litter - any persistent, manufactured or processed solid material- is found lying on the shores, as well as floating anywhere from the surface to the bottom of the sea. Even in pristine environments of the Mediterranean, such as coastal and marine protected areas (MPAs), marine litter is building up, threatening habitats and species. Impacts vary from entanglement and ingestion, to bio-accumulation and bio-magnification of toxic substances released from litter items, facilitation of introduction of invasive species, damages to benthic habitats, etc. MPA managers stand at the forefront of this issue, and admittedly they lack the tools, knowledge, and often the resources to effectively tackle it. As a result, the achievement of the conservation goals set is hampered.

Figure 1-1. *Marine litter a lurking threat in Mediterranean MPAs (Photo © Th. Vlachogianni).*

1.2 The Plastic Busters MPAs project in a nutshell

The 4-year-long Interreg Med Plastic Busters MPAs project aimed at contributing to biodiversity protection and preservation of natural ecosystems in pelagic and coastal marine protected areas (MPAs), by defining and implementing a harmonized approach against marine litter. The project entailed actions that addressed the entire management cycle of marine litter, from monitoring and assessment to prevention and mitigation, as well as actions to strengthen networking between and among pelagic and coastal MPAs.

Plastic Busters MPAs consolidated Mediterranean efforts against marine litter by:

- Assessing the impacts of marine litter on biodiversity in MPAs and identifying marine litter ‘hotspot’ areas;
- Defining and testing tailor-made marine litter surveillance, prevention and mitigation measures in MPAs;
- Developing a common framework of marine litter actions for Interreg Mediterranean regions towards the conservation of biodiversity in Mediterranean MPAs.

The Plastic Busters MPAs project deployed the multidisciplinary strategy and common framework of action developed within the Plastic Busters initiative led by the University of Siena and the Sustainable Development Solutions Network Mediterranean (SDSN Med). This initiative frames the priority actions needed to tackle marine litter in the Mediterranean basin and was labelled under the Union for the Mediterranean (UfM) in 2016, gathering the political support of 43 Euro-Mediterranean countries.

Figure 1-2. *The Plastic Busters MPAs project in a nutshell.*

1.3 Definitions and policy context

Within this document, marine litter is defined as any persistent, manufactured or processed solid material discarded, disposed of, or abandoned in the marine and coastal environment. Marine litter can be classified in size classes as follows: macrolitter refers to items larger than 25 mm in the longest dimension, mesolitter to items between 5 mm to 25 mm, and microlitter to items ranging from 1 µm to 5 mm. This latter size class is sometime further broken down into large microlitter ranging from 1 mm to 5 mm and microplastic, from 1 µm to 1 mm in size.

The main legislative frameworks related to marine litter monitoring are the EU Marine Strategy Framework Directive – MSFD (2008/56/EC, 2010/477/EC, 2017/848/EC) and the Barcelona Convention Ecosystem Approach (COP19 IMAP Decision IG.22/7, UNEP/MED WG.450/3, June 2018) (see Box 1.1 and Box 1.2).

Box 1.1. *The Marine Litter Descriptor, criteria, and respective Indicators within the framework of the EU MSFD.*

Marine Litter within the EU MSFD

Descriptor 10: ***Properties and quantities of marine litter do not cause harm to the coastal and marine environment***

Criteria D10C1 - Primary: The composition, amount and spatial distribution of litter on the coastline, in the surface layer of the water column, and on the seabed are at levels that do not cause harm to the coastal and marine environment.

- amount of litter washed ashore and/or deposited on coastlines, including analysis of its composition, spatial distribution and, where possible, source (10.1.1)
- amount of litter in the water column (including floating at the surface) and deposited on the seafloor, including analysis of its composition, spatial distribution and, where possible, source (10.1.2)

Criteria D10C2 - Primary: The composition, amount and spatial distribution of micro-litter on the coastline, in the surface layer of the water column, and in seabed sediment are at levels that do not cause harm to the coastal and marine environment.

- amount, distribution and, where possible, composition of microparticles (in particular microplastics) (10.1.3)

Criteria D10C3 - Secondary: The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned.

- amount and composition of litter ingested by marine animals (10.2.1)

Criteria D10C4 - Secondary: 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.

Box 1.2. *The Marine Litter Operational Objectives and respective Indicators within the framework of the Barcelona Convention Ecosystem Approach and the Integrated Monitoring and Assessment Programme (IMAP).*

Marine Litter and the Barcelona Convention Ecosystem Approach

Ecological Objective 10 (EO10): Marine and coastal litter do not adversely affect the coastal and marine environment.

IMAP Common Indicator 22:

Trends in the amount of litter washed ashore and/or deposited on coastlines (including analysis of its composition, spatial distribution and, where possible, source).

IMAP Common Indicator 23:

Trends in the amount of litter in the water column including micro plastics and on the seafloor.

IMAP Candidate Indicator 24:

Trends in the amount of litter ingested by, or entangling marine organisms, focusing on selected mammals, marine birds, and marine turtles.

1.4 About this document

The overarching aim of this document is to provide an operational protocolfor implementing the Plastic Busters MPAs harmonized marine litter monitoring approach and assess the presence and effects of marine litter in pelagic and coastal Mediterranean MPAs with special emphasis on marine species, including endangered ones (cetaceans, sea turtles, birds, endangered sharks, etc.). In this respect, this document is a compilation of all the protocols that should be applied in order to elaborate a comprehensive diagnosis of the marine litter problem in Mediterranean MPAs.

This document takes stock of all recent advances made by the EU MSFD Technical Group on Marine Litter and the Barcelona Convention CORMON Group. Furthermore, this document capitalizes on the outcomes of relevant projects such as the IPA-Adriatic DeFishGear project, the EU-funded INDICIT project and the Interreg Med marine litter related projects, namely the MEDSEALITTER, AMARE and ACT4LITTER.

Photo © Th. Vlachogianni

10. Monitoring presence and impact of marine litter in biota: the Plastic Busters MPAs approach

This document describes the methodological approach for monitoring the presence and impact of Marine Litter in the Mediterranean Biodiversity (i.e., the Plastic Busters MPAs Protocols), which has been developed within the framework of the Interreg Med Plastic Busters MPAs project, building on the most recent methodological advances of the MSFD TGML, INDICIT II Project, Barcelona Convention CORMON, and on the results of the project's testing phase.

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10.1 Monitoring the presence and effects of marine litter in biota: the Plastic Busters MPAs approach

This introductive chapter describes the comprehensive methodological approach, developed within the framework of the Interreg Med Plastic Busters MPAs project, for monitoring the presence and effects of marine litter in the biodiversity inhabiting Mediterranean MPAs. This approach has been developed in order to evaluate the impact (mainly ingestion) of marine litter on both **commercially harvested** (invertebrates and fish) and **endangered species** (cetaceans, sea turtles, seabirds).

The Plastic Busters MPAs methodological approach builds on the methodologies developed by the MSFD TGML, the Barcelona Convention CORMON, the IPA-Adriatic DeFishGear project, the Interreg Med MEDSEALITTER project, the INDICIT II project, taking into account the findings of the testing phase of the Interreg Med Plastic Busters MPAs project. The resulting protocols are characterized by two main novelties:

- The selection of a wide range of bioindicator species;
- The development of a new diagnostic tool: the threefold monitoring approach.

10.2 The marine litter impact on biodiversity: candidate biondicators selection

The selection of sentinel species to monitor the impact of marine litter on Mediterranean fauna is a crucial step for the development of harmonized sampling methods and protocols for the establishment of a consistent regional approach at Mediterranean basin scale. The selection of sentinel species, or "*candidate bioindicators*", has to meet specific criteria and respond to the need of monitoring different habitats in Mediterranean MPAs (from coastal areas to offshore, from benthic environments to pelagic waters) at different spatial scales, and home ranges (Fossi et al. 2018).

Several sentinel species are proposed in this protocol as "candidate bioindicators" to detect the presence and impact of marine litter; they have been identified according to: 1) available data on marine litter interactions with Mediterranean marine organisms, 2) key ecological and biological criteria for selecting sentinel species, 3) key outcomes of previous projects and, 4) the main finding of the testing phase of the Plastic Busters MPAs project.

One of the main parameters considered is the marine litter (ML) **% of occurrence**: the various species analyzed are reported according to three different ranges (classes) of ML occurrence: *Low ML occurrence (0-30%), Medium ML occurrence (31-60%), High ML occurrence (61-100%)*.

This finding allows to select species with the highest ingestion rate (reported in Table 10.1) in addition with other key ecological and biological criteria such as:

- **Home range**: local scale, small-scale (FAO Geographical subareas), medium-scale (Mediterranean UN Environment/MAP sub-regions) and Mediterranean Basin scale.
- **Habitat**: sea surface, coastal waters, open waters, seafloor.
- **Distribution** in targeted Mediterranean MPAs.
- **Frequency of Occurrence** of ingestion of marine litter (in bold species with % of marine litter occurrence >30%)

Table 10-1. ML Candidate Bioindicators *proposed for each habitat, ecological compartment and home range (in **bold** species with % of marine litter occurrence >30%, * species studied in the Plastic Busters MPAs project).*

	SEA SURFACE	COASTAL WATERS	OPEN WATERS	SEAFLOOR
BASIN SCALE ***(Mediterranean Sea)***		***Puffinus yelkouan****	***Balaenoptera physalus**** ***Calonectris diomedea**** ***Mobula mobular**** ***Physeter macrocephalus**** *Thunnus thynnus* *Xiphias gladius*	
MEDIUM-SCALE ***(Mediterranean UN Environment/MAP sub-regions)***		***Tursiops truncatus****	***Caretta caretta**** ***Chelonia mydas**** ***Coryphaena hippurus*** ***Dermochelys coriacea*** ***Globicephala melas**** ***Stenella coeruleoalba**** ***Ziphius cavirostris**** *Grampus griseus** *Thunnus alalunga*	
SMALL-SCALE ***(FAO GSA)***	***Velella velella**** *Isopods*	***Boops boops**** ***Monachus monachus**** ***Oblada melanura**** ***Serranus cabrilla**** ***Serranus scriba**** ***Spicara smaris**** ***Spondyliosoma cantharus**** *Trachurus trachurus**	***Engraulis encrasicolus**** ***Sardina pilchardus**** *Myctophium punctatum* *Scomber sp.*	***Epinephelus marginatus**** ***Diplodus anularis**** ***Diplodus vulgaris**** ***Lithognathus mormyrus*** ***Merluccius merluccius**** ***Mullus surmuletus**** ***Pagrus pagrus**** *Galeus melastomus* *Mullus barbatus**
LOCAL SCALE		***Arca noae**** ***Modiolus barbatus**** ***Mytilus galloprovincialis ****		***Holothuria forskali**** ***Holothuria poli**** ***Holothuria tubulosa**** *Arbacia lixula** *Paracentrotus lividus**

In Table 10-1, the "candidate bioindicators" for each habitat and ecological compartment have been reported, also in light of the main finding from the testing phase of the Plastic Busters MPAs project . The ML candidate bioindicators are proposed according to their different frequency of occurrence, which can be used as a proxy for exposure to ML ingestion *(Medium ML occurrence (31-60%), High ML occurrence (61-100%)* (Fig. A1, Annex I).

10.3 The threefold monitoring approach

Assessing the impact of litter on marine organisms is a challenging task. Physical and ecotoxicological effects strictly related to marine litter and, in particular, to plastics can be directly addressed in just few cases, thus calling for an integrated approach. The impact of litter on marine organisms should be assessed using a multi-tier approach, tested within the Plastic Busters MPAs project, which links marine litter ingestion detection with the physical and toxicological effects related to the ingestion of of contaminated plastic litter and the contaminants absorbed on litter and the leaching chamicals (e.g additives .. The application of the threefold approach, described in the next chapters, can elucidate not only the rate of ingestion among the different bioindicators, but also the multiple sub-lethal stresses that marine litter ingestion can cause in the short and long term. Each of the three investigation tools that make up the threefold approach can be applied independently or simultaneously to the selected candidate bioindicators. Sentinel species are subdivided into two categories: a) **commercially harvested species**; and b) **endangered species** (free-ranging and stranded marine mammals, hospitalized and stranded sea turtles) (Fig. 10.1).

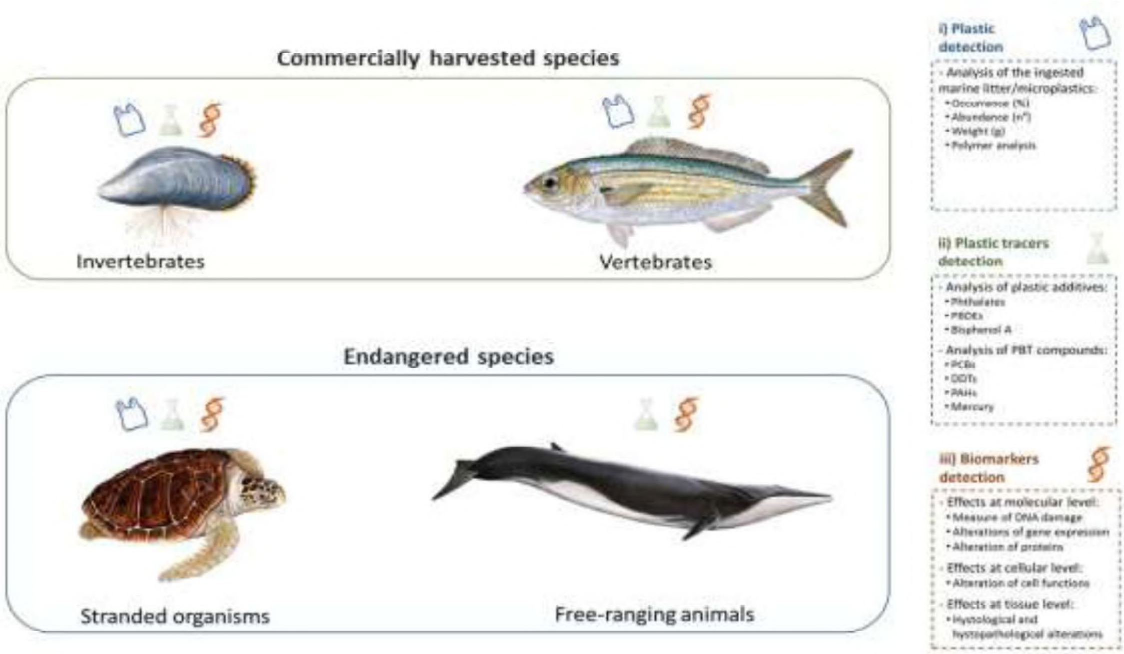


Figure 10-1. *The threefold monitoring approach applied in the Plastic Busters MPAs project.*

The implemented monitoring approach – defined as the *threefold monitoring approach* – relies on the following three types of data:

I. **analysis of the gastro-intestinal content** in vertebrates/invertebrates (or of the whole organism, in the case of small invertebrates) to evaluate the marine litter ingested by the selected species, with a particular focus on plastics and microplastics. This analysis must focus on assessing the occurrence (%) of individuals that have ingested marine litter, the abundance (n° of items) of marine litter ingested per individual, the weight (g) of marine litter ingested as a total and per category of litter, the colour of litter items, as well as the polymer characterization of the plastic litter and microplastics ingested by the different individuals/species analyzed. Information on the extent to which marine biota ingests marine litter (including microplastics) is essential to determine threshold levels to define 'good environmental status' (GES) for marine litter and plastic pollution (as recommended by the EU MSFD and other regional and international regulations, i.e. Descriptor 10 of theMSFD, Ecological Objective 10 of the Barcelona Convention Ecosystem Approach).

II. **quantitative and qualitative analyses of plastic additives** (e.g., phthalates and polybrominated diphenyl ethers-PBDEs) and Persistent, Bioaccumulative and Toxic (PBT) compounds in the tissues of bioindicators, used as "plastic tracers". The detection of plastic additives and PBT compounds that can be transferred from plastic litter to the tissues of organisms could represent the degree of accumulation of compounds related to the ingested plastic litter and the causes of its putative ecotoxicological effects. The evaluation of plastic tracers, especially in biological materials obtained in a non-lethal way in endangered species (e.g. skin biopsies), can represent a proxy of ingestion of plastic materials.

III. **analysis of the effects of marine litter and additives based on biomarker responses at different biological levels** (from gene/protein expression variations to histological alterations; Omics techniques). Assessing the biological responses (alteration of a set of biomarkers by the measurement of endpoints) to the ingestion of marine litter and the accumulation of plastic associated compounds is crucial; this allows understanding and evaluating the extent to which marine litter and plastic ingestion pose a threat to marine organisms at individual and, ultimately, population level.

The application of the three categories of monitoring techniques (Fig. 10.2) – i) Marine litter ingested detection, ii) Plastic tracers' detection, and iii) Biomarkers detection in the candidate bioindicators– requires varying degrees of expertise, ranging from techniques easily applicable by the majority of institutions involved in marine litter monitoring (marine litter ingested detection), to the most specialized and complex ones, such as the estimation of ecotoxicological effects (plastic tracers, biomarker and Omics analysis). The gradient of expertise is described below for four typologies of organisms: a) commercially harvested species, b) stranded endangered species, c) hospitalized endangered species, d) free-ranging endangered species.

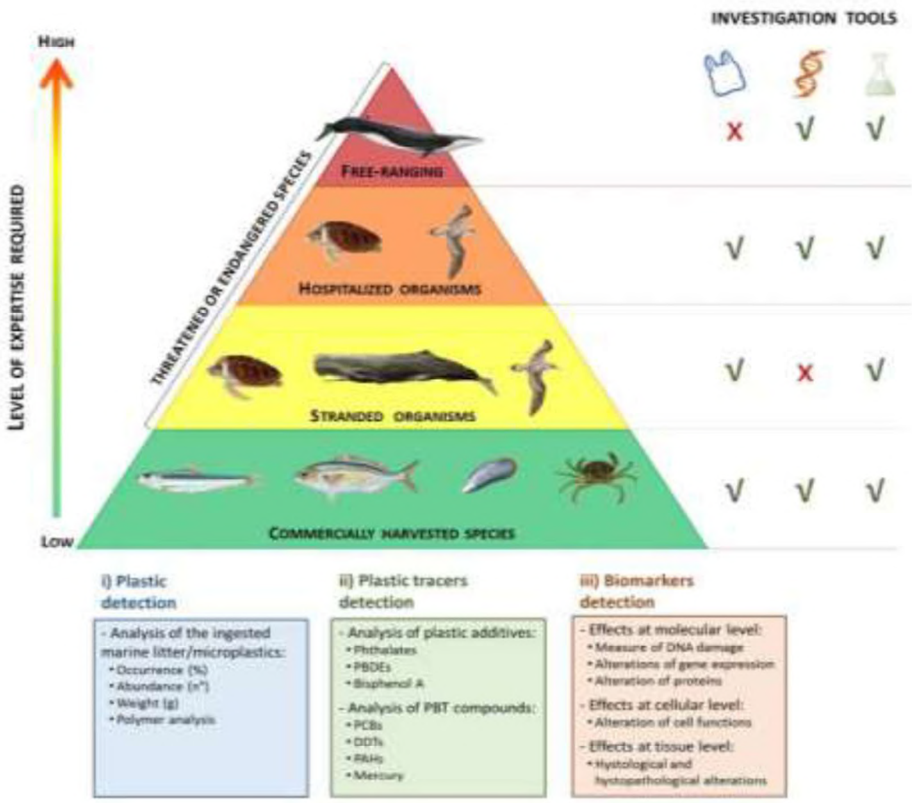
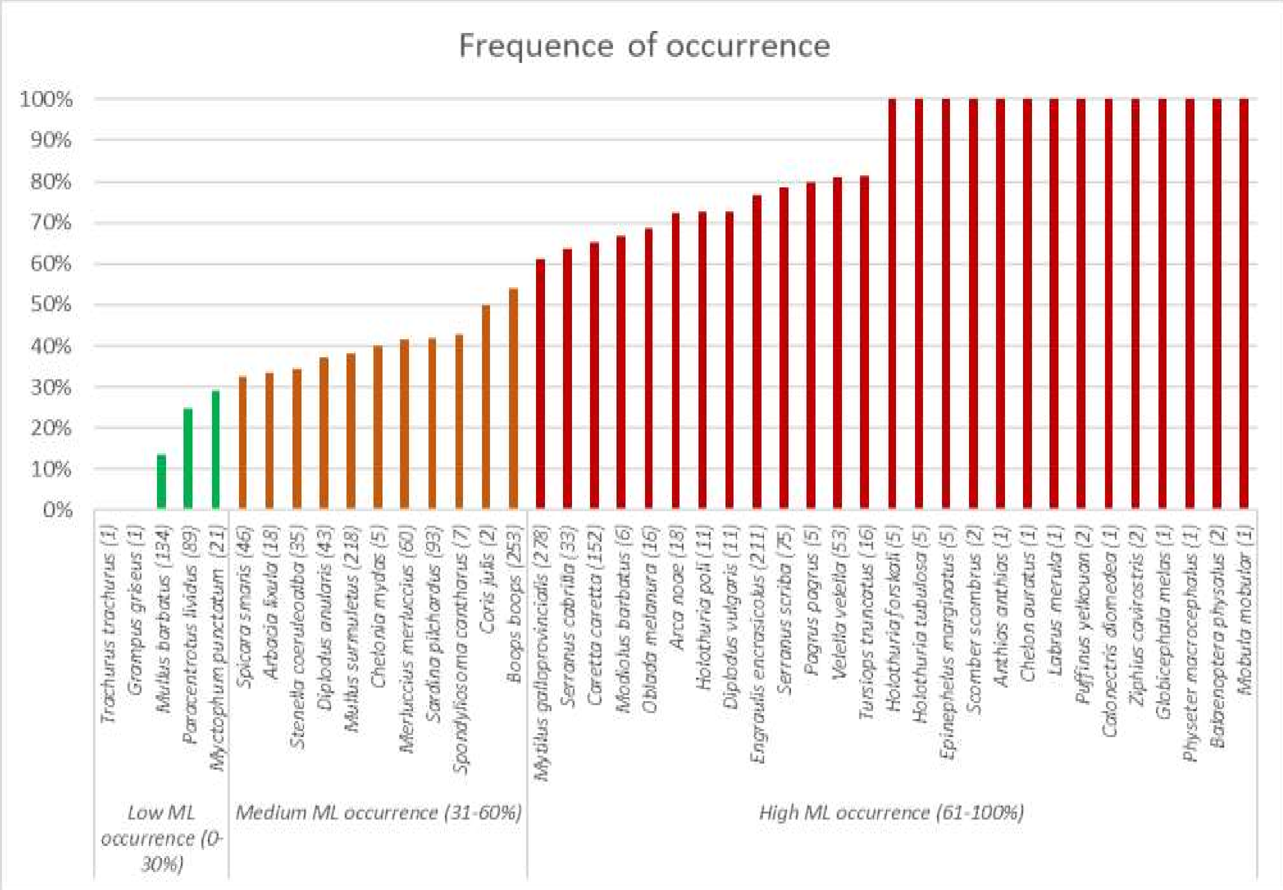


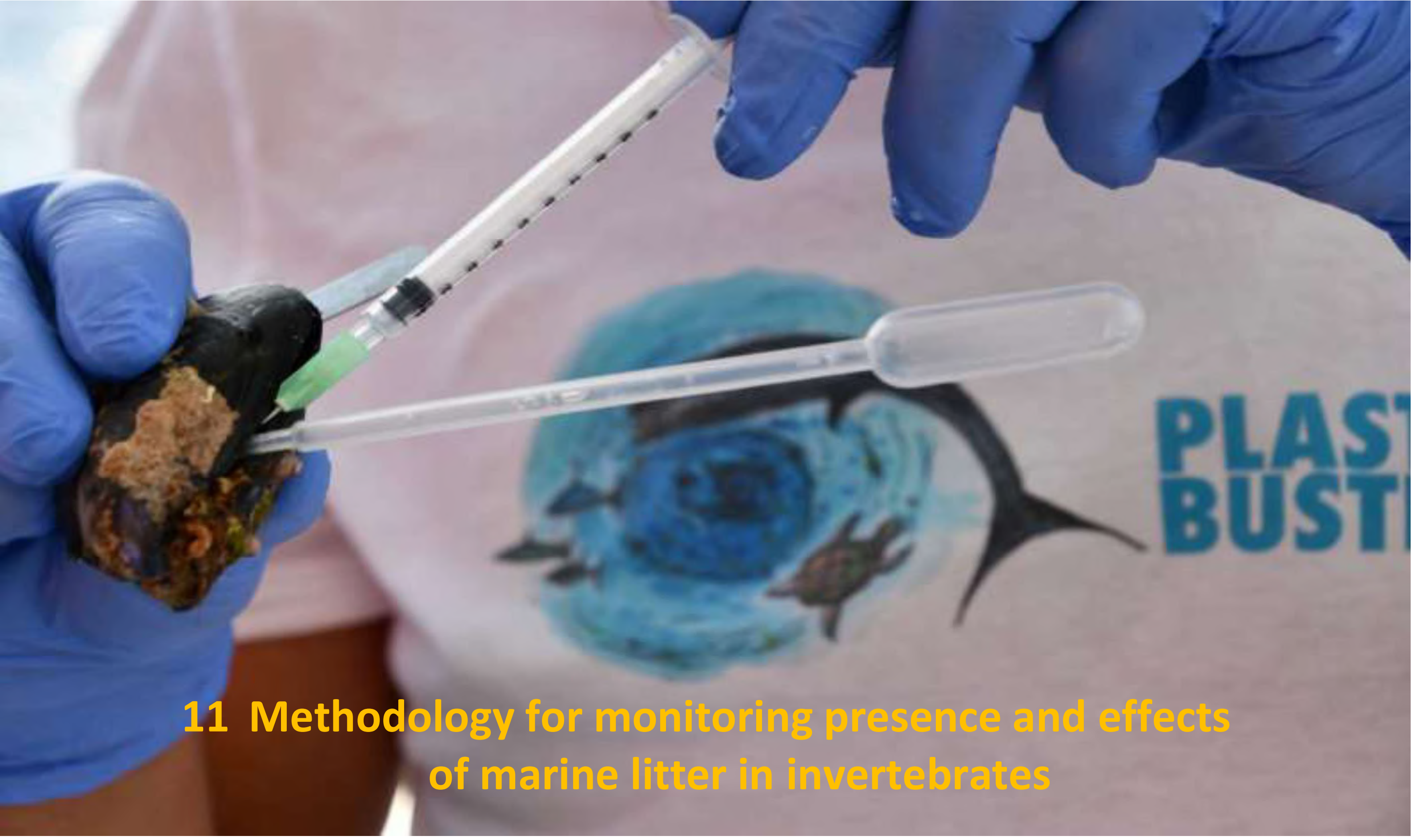
Figure 10-2. *Level of expertise required for the detection of marine litter ingestion and impact in Mediterranean biota as adopted by the Plastic Busters MPAs project. Blue plastic bag: marine litter detection; DNA double helix: biomarker detection; green flask: contaminants (plastic tracers) detection.*

ANNEX I

Figure A-1. *ML (including MPs) frequency of occurrence in the 46 selected biondicators, ranging in term of % of frequency of occurrence: Green Low ML occurrence (0-30%), Orange Medium ML occurrence (31-60%), Red High ML occurrence (61-100%).*



In order to propose a series of candidate bioindicator species to identify the presence and impact of marine litter in Mediterranean MPAs, the various species analyzed in the testing phase of the Plastic Busters MPAs project, are reported according to three different range of ML occurrence: *Green Low ML occurrence (0-30%), Orange Medium ML occurrence (31-60%), Red High ML occurrence (61-100%).* (Figure 1A). This data will subsequently be used to select the species with the highest ingestion rate which will be reported in Table X.1 in addition with other ecological and biological parameters.



11 Methodology for monitoring presence and effects of marine litter in invertebrates

This document describes the methodological approach for monitoring the presence and effects of marine litter in invertebrates, which has been developed within the framework of the Interreg Med Plastic Busters MPAs project, building on the most recent methodological advances of the MSFD TGML and Barcelona Convention CORMON, and on the results of the project's testing phase.

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11.1 Sampling approaches

Marine invertebrate species such as filter-feeding invertebrates (e.g., mussels), and other invertebrate species (e.g., sea urchins) should be collected following any of the modalities below:

- Marine invertebrates are collected inside the study area.
- Marine invertebrates are collected in adjacent areas with similar conditions and are re-located in the study area with the use of metal cages. After a period of 3-4 weeks, they can be sampled.
- Marine invertebrates are purchased by local fishers active in the study area.

It is recommended to record the following information for each sampling site:

- **Climate variables**: Sea temperature (in °C), mean wave height, maximum wave height, mean wave period, wave direction, etc.
- **Environmental variables:** Sediment granulometry, nutrients, turbidity, chlorophyll-a, salinity, etc.
- **Habitat Characteristics:** Habitat type (e.g., sand, seagrass, algae, mats), habitat composition (% seagrass, % sand), etc.
- **Coastline morphology:** Beach, cliffs, estuaries, closed bay, open bay, creeks, etc.
- **Anthropogenic variables:** Anchoring allowance, diving, sewage input, fishing activities, presence of fishing gear, poaching, etc.
- **Protection status:** Protection level (fully protected, partially protected, not protected), protection status (e.g., Marine Reserve, Natural Park, Site of Community Importance), number of years before/after the establishment of protection status, etc.

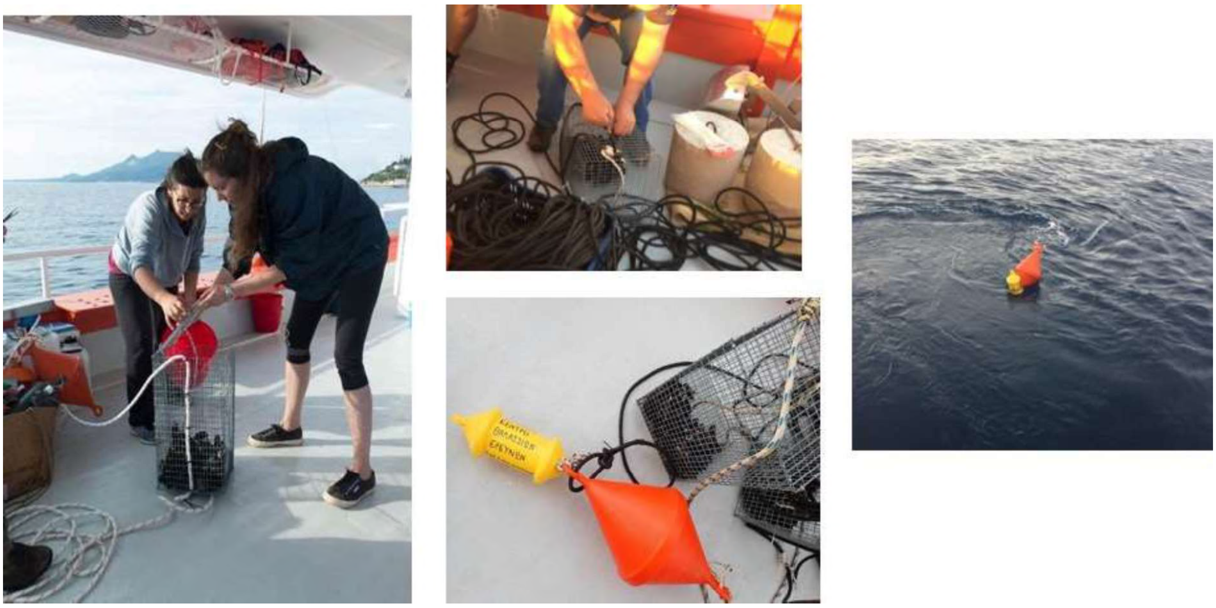


Figure 11-1. *Sampling approach using translocated mussels in metal cages.*

For specimens purchased from fishers the following information should be recorded: the date and time of capture, the name of the boat(s) and fishing gear used, the sampling depth. If possible, the latitude and longitude of each point where the species were captured should be recorded. If this is not possible, the area where the species were captured could be extrapolated from the Automatic Identification System (AIS).

11.2 Frequency and timing of sampling

Marine invertebrates should be sampled at least once per year.

11.3 Sample size

Irrespective of the chosen sampling approach (from those listed above), the minimum number of specimens sampled per sampling site should be as follows:

- Mussels: 30 specimens
- Sea urchins: 30 specimens

***Figure 11-2**. Sea urchins sampling.*

11.4 Tissues collection

To perform litter, contaminants and biomarker analyses, tissues should be removed from living organisms. Alternatively, if performing only litter and contaminant analyses, tissues can be dissected from animals frozen at -20 °C. Before the dissection of the specimens, the following information should be recorded:

- The name of the species.
- The weight of each individual (removing byssus filaments for mussels, accurate to the 4^{th} decimal per individual).
- The length and width of each individual.
- Any visible deformations.
- The standard identification code of the animal written on the label.

Once in the laboratory, proceed either with the dissection of tissues for microplastics analysis and contaminant analysis or for biomarkers analysis or store the specimen at -20 °C or -80 °C until future dissection for microplastics or biomarkers analyses, respectively.

Dissect the following tissues (to be labelled with a unique ID for each individual):

- **Hemolymph (mussels), coelomic fluid (sea urchins)**: hemolymph should be withdrawn from the adductor muscle of mussels using a disposable heparinized syringe with a 23G or 18G needle. The coelomic fluid should be drawn from sea urchins by a syringe inserted in the peristomal membrane around the Aristotele's lantern. Use part of the haemolymph or coelomic fluid to obtain smears and an aliquot for different biomarkers analysis (stored at -80°C).
- **Digestive gland (mussels), gastrointestinal tract (sea urchins)**: it should be collected and weighted in aluminium paper, placed in labelled cryogenic vials, frozen in liquid nitrogen and stored at -80 °C or dry ice for biomarkers analysis. For microplastic analysis, it should be placed in aluminium foil and stored at -20 °C.
- **Gills (mussels):** they should be collected, placed in labelled cryogenic vials, frozen in liquid nitrogen and stored at -80°C or dry ice for biomarkers analysis. For microplastic analysis, they should be placed in aluminium foil and stored at -20 °C.
- **Mantle (mussels)** (Fig. 11.3)**:** it should be collected for biomarkers analyses, frozen in liquid nitrogen and stored in labelled cryogenic vials at -80 °C or dry ice. For microplastic analysis, they should be placed in aluminium foil and stored at -20 °C.
- **Gonads (mussels, sea urchins):** they should be collected for biomarker analyses, part of the tissue placed in labelled cryogenic vials, frozen in liquid nitrogen and stored at -80 °C or dry ice and another part stored in Bouin's solution.

If the dissection of the different tissues is not possible, the whole organism should be used for litter analyses.

Whole organisms should be stored in aluminium foil for contaminant analyses.

***Figure 11-3**. Dissection of mussel.*

11.5 Litter size classes to be surveyed

The litter size classes to be surveyed depend on the size of the investigated invertebrate. Usually for mussels and small size invertebrates, only large and small microplastics are ingested and can be detected. Litter items with their longest dimension larger than 50 µm can be detected using the protocol described below for microplastic analyses.

11.6 Litter analysis, classification and quantification

Once at the laboratory, biological samples should be first digested, then sorted and identified under a stereomicroscope with optical enhancement from 6.7x to 40.5x (Alomar et al., 2016, Nadal et al., 2016) following the protocol described in Tsangaris et al. (2021), which is the result of an intercalibration among Plastic Busters MPAs project partners.

Microplastic analysis

- Place the tissue sample in a glass beaker, add 5 ml 10% KOH per gram of tissue wet weight (1:5 w/v).
- Cover the samples with aluminum foil and heat them up on a thermostatic water bath (50 °C) until all organic matter is removed (maximum 2 days, 12 hours heating).
- After the digestion of the organic matter, pass the samples through a metal sieve (300 µm) placed above a filtering apparatus and finally filter the sample on a fiber glass filter under vacuum (Whatman GF/C, pore size 1.2 or 1.6 µm).
- Metal sieves shall be covered with aluminium foil and filters shall be placed in aluminium foil-covered Petri dishes and dried at room temperature.
- All filtering procedures shall take place inside a laminar flow cabinet.
- Use a procedural blank sample to test for possible ambient contamination: add similar volume of 10% KOH as that used in the samples in a beaker without sample, and follow the protocol described in the steps above.
- After the digestion procedure, check the filter for plastic items with the use of a stereomicroscope.
- Photograph, count and record the type, colour and maximum length of plastic particles using an image analysis software. Categorize plastic particles according to shape, size, colour and polymer
- Additionally, 10% of the identified items should be considered for identification using spectroscopy techniques (FT-IR, RAMAN).
- The recovery rate of microplastics by the applied extraction procedure must be tested on tissue samples enriched with specific number (e.g. 10 particles/sample) of different types of plastic particles. Use the number of particles detected after processing the sample to calculate % recovery rate of microplastics.

Contamination precautions

Contamination precautions are essential during all steps of the sample processing due to the ubiquitous nature of certain types of microplastics, such as synthetic fibers, that can contaminate the samples. Glass material should be used where possible and all glassware and tools (e.g. tweezers, scissors, etc.) should be rinsed thoroughly with purified water. Staff should wear natural fiber laboratory clothes. Sample processing should be done in closed areas with little ventilation and air circulation (e.g., from air conditioners). It is recommended to use covers during sample rinsing and filtration (e.g., glove bag, laminar flow cabinet or other closed cover) and to cover filters with glass lids during observation under the stereomicroscope. Procedural blank samples should be used throughout the entire sample processing. During the analysis procedure, two glass petri dishes should be placed at each side of the stereomicroscope and checked for microplastics before and after each sample. A 100% cotton laboratory coat shall be worn at all times during the procedure.

Figure 11-4. *Main steps of invertebrate sample processing for microplastic detection (from Tsangaris et al. 2015).*

Collection of data

For each species an assessment is made of:

1. Frequency of occurrence (%) of ingested microplastics, calculated as the percentage of the individuals examined with ingested microplastics.
2. Abundance (N) of macro and microplastics ingested per individual (average number of items/individual) for each species, calculated as a total and per category. Given currently existing inconsistencies in the literature regarding reporting the abundance of ingested litter, it is recommended to report average number of items per individual, considering both all the individuals examined and those solely found with ingested litter.

For each individual organism, the following information on ingested litter shall be reported:

1. The number, length, weight and nature of the polymer (10%) of the items examined.
2. The recovery rate of microplastics.

11.7 Analysis of plastic tracers and PBTs

Plastic additives

The compounds to be detected are:

- *Phthalates:* a group of chemicals widely used (e.g., plastic additives) to make plastics more flexible and harder to break; they can interfere with the endocrine system (Baini et al., 2017).
- *Bisphenol A*: used in the production of polycarbonate, can have endocrine disrupting effects (Crain et al., 2007; Halden, 2010; Oehlmann et al., 2009) and the styrene and polyvinyl chloride monomer, used in the production of polystyrene and polyvinyl chloride (PVC), can be carcinogenic and/or mutagenic (Lithner et al., 2011; Papaleo et al., 2011; Xu et al., 2004).

- *Polybrominated diphenyl ethers:* they belong to the group of brominated flame retardants (BFRs), which are used in various polymeric materials (e.g., plastic parts, resins, textiles, and other substrates) to reduce their fire hazards (BSEF 2003; Król et al. 2012).

Persistent, bioaccumulative and toxic substances (PBTs)

In addition to the plastic additives that may leach from the plastic items released into the marine environment, persistent bioaccumulative and toxic substances (PBTs) (e.g. organochlorine compounds OCs, PAHs and PBDEs) and metals (e.g., lead, copper and cadmium) that are present in the seawater tend to accumulate in the surface of plastic items.

Depending on the compounds and the tissues to be analysed, different methods should be applied to detect the presence of plastic-related contaminants in invertebrates (Annex II, Table A.1).

11.8 Biomarkers analysis

The toxicological effects associated with the presence of marine litter can be evaluated using a set of diagnostic and prognostic methodologies, by means of biomarkers. A non-exhaustive list of existing biomarker approaches and plastic tracers' contaminants that are usually applied in invertebrates analyses is reported in Annex II (Table A-2) and Figure 11.5.

Biomarkers have been selected on the basis of the level of biological responses and in relation to the main effects related to marine litter/microplastics ingestion. The selected biomarkers can diagnose different impacts related to: a) physical damages/effects of marine litter, b) exposure to/effect of chemical tracers, and c) exposure to/effect of adsorbed chemicals.

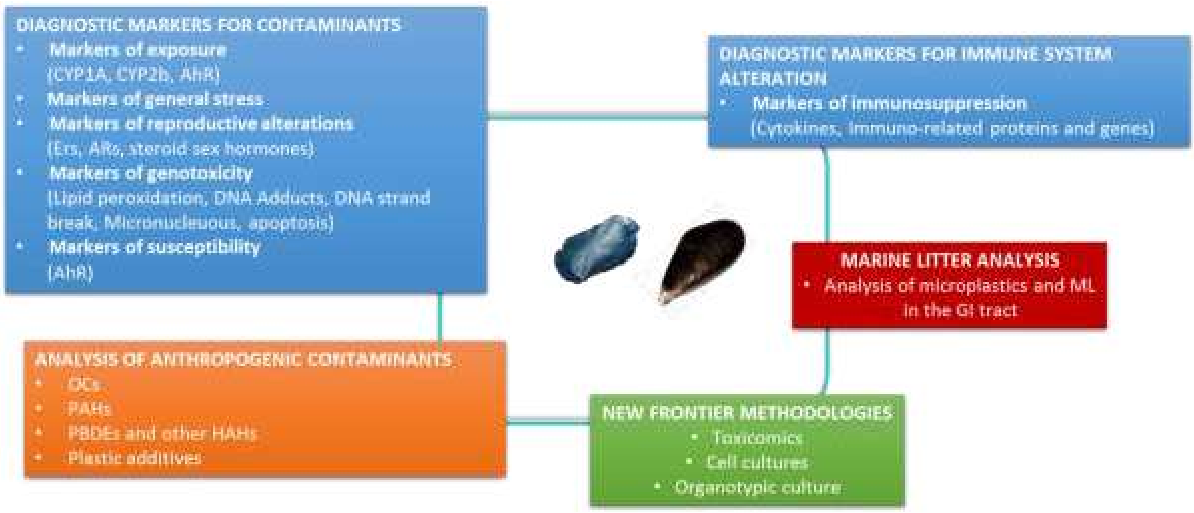
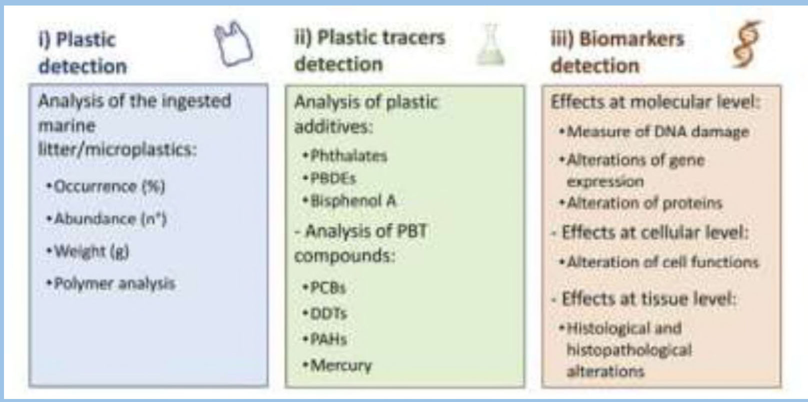


***Figure 11-5**. A three-fold approach to detect the marine litter presence and impacts to invertebrates.*

Starting from this initial list and building on the findings of the testing phase of the Plastic Busters MPAs project, the most suitable diagnostic tools to detect the presence and impact of ML on invertebrates are proposed here below.

Table 11-1. Main diagnostic tools selected in the Plastic Busters MPAs project to detect the presence and impact of ML in invertebrates.

INVERTEBRATES: MAIN DIAGNOSTIC TOOLS SELECTED IN THE PLASTIC BUSTERS MPAs PROJECT



1. Analysis of microplastic ingestion
2. Analysis of plastic tracers: Phthalates
3. Analysis of biological end-points: MN assay, lysozyme, LPO, gene expression (*il17r, hsp70, tlr2, nf-kb, mt10, idh, clhc1*)

11.9 Materials & Equipment

Material for sampling

- Camera
- Containers for samples, zipped bags, cool boxes
- Garbage bag
- Gloves
- Disposable scalpels, fine forceps, scissors and tweezers for dissection
- Dissection board
- Measuring decimetre
- Pen/pencil/ Permanent marker
- Sampling sheets
- Aluminium foil
- Cryoboxes
- Cryovials
- Liquid nitrogen Dewar (in alternate dry ice)
- Paper and block-notes
- Paper towels

Material for microplastic analysis

- Distilled water
- Permanent marker
- 10% KOH
- Disposable scalpels, fine forceps, scissors and tweezers for dissection

- Precision tweezers (fine and pointed) for micro-plastic handling on filters and FTIR
- Glass petri dishes
- GFC filters 0.2, 1.2 or 1.6 µm 47 mm diameter for filtration
- Aluminum foil
- 150 and 250 ml glass beakers
- 250 ml conical flasks
- 100 ml measuring glass cylinder
- Magnetic stirrer
- Hot plate
- Glove bag, laminar flow cabinet or other closed cover
- Vacuum filtration system with ramp
- Analytical balance
- Stereomicroscope with image analysis software
- FTIR or Raman spectroscopy with associated analysis software

References

Guidance on Monitoring of Marine Litter in European Seas, 2022. A guidance document within the Common Implementation Strategy for the marine Strategy Framework Directive - Marine Litter Impact on Biota. MSFD Technical Group on Marine Litter - draft.

Fossi, M.C, Vlachogianni, T., Anastasopoulou, A., Alomar, C., Baini, M., Caliani, I., Campani, T., Casini, S., Consoli, P., Cillari T., D'Alessandro, M., Deudero, S., Galgani, Galli M., F., Kaberi H., Panti, C., Pedà, C., E. Romeo, T., Scotti, G., Tsangaris, C., Zeri, C., 2019. Toolkit for monitoring marine litter and its impacts on biodiversity in Mediterranean MPAs. Interreg Med Plastic Busters MPAs project

11.10 Sampling & recording sheets

Monitoring Microlitter in biota: mussels

Sampling date and time	Sampling site	GSA	Sampling method	Depth	Coordinates	
					Latitude	Longitude

ID code	Species	Sex	Shell length (cm)	Shell weight (g)	Soft tissue weight (g)	Digestive gland weight (g)	Gills	Hemolymph		Mantle
								MN test	(ul)	

Monitoring Microlitter in biota: sea urchins

Sampling date and time	Sampling site	GSA	Sampling method	Depth	Coordinates	
					Latitude	Longitude

ID code	Species	Sex	Weight (g)	Diameter (cm)	Soft tissues weight (g)

Notes and remarks:

ANNEX II

Table A-1. *Tissues and methods to be used to detect plastic tracers in invertebrates.*

	CHEMICAL COMPOUND	TISSUE/SAMPLE	ANALYTICAL METHOD
PLASTIC ADDITIVES	**Phthalates**	Muscle, whole organism	Baini et al., (2017), Fossi et al., (2016), Savoca et al., (2018), Avisar et al., (2019), Lo Brutto et al., (2021),
	Bisphenol A	Muscle, whole organism	Ballesteros-Gómez et al., (2009), Lo Brutto et al., (2021)
	Polybrominated diphenyl ethers	Muscle, whole organism	Muñoz-Arnanz et al., (2016), Cruz et al., (2019), Cruz et al., (2020)
ADSORBED CONTAMINANTS	**Polycyclic aromatic hydrocarbons**	Muscle, whole organism	Marsili et al., (2001), León et al., (2013), Benedetti et al., (2014)
	Organochlorine contaminants	Muscle, whole organism	Marsili and Focardi, (1997), León et al., (2021)
	Mercury	Whole organism, muscle	Correa et al., (2013), Fattorini et al., (2008), Besada et al., (2011), León et al., (2021)

Table A-2. *Effects measured in invertebrates by the biomarker approach.*

EFFECT	TISSUE	TEST
GENOTOXICITY	Hemolymph, digestive gland	Comet assay (Revel et al., 2019) (*) Mn test (Avio et al., 2015) (*)
OXIDATIVE STRESS	Digestive gland	LPO, CAT, SOD, GST, GSH, GR, GPX (Revel et al., 2019) (*) qPCR GPX, SOD, CAT (Ravel et al., 2019)
IMMUNOTOXICITY	Gills, Mantle, Digestive gland	CASP, TRAF, Transcriptomics (Avio et al., 2015; Revel et al., 2019) (*) Transcriptomics (Gardon et al., 2020) qPCR LYS, CASP3 (Paul-Pont et al, 2016)
REPRODUCTION	Gonads	Gamete Quality and Larval Development (Sussarellu et al., 2016) (*)
HISTOPATHOLOGY INFLAMMATION AND MORPHOLOGY	Digestive gland	Histopathology, histology (Avio et al., 2015) (*)
XENOBIOTIC METABOLISM AND BIOTRANSFORMATION	Digestive gland, whole organism	Porphyrins (Grandchamp et al. 1980; Guerranti et al. 2014) (*) EROD (Zhang et al., 2019) Transcriptomics (Gardon et al., 2020) (*)
NEUROTOXICITY	Whole organisms, muscle, gills	AChE activity (Magni et al., 2018) (*)
CELLULAR STRESS	Whole organisms, muscle, hemolymph, digestive gland	Lysosomal membrane stability-LMS (Canesi et al 2015) (*) IDH (Oliveira et al., 2013) (*) Transcriptomics (Détréé et al. 2018) qPCR IDH, HSP70 Détréé et al. 2017)

(*) effects detected after laboratory or field exposure with MPs or plastic-related contaminants.

12 Methodology for monitoring presence and effects of marine litter in fish

This document describes the methodological approach for monitoring the presence and effects of marine litter in fish, which has been developed within the framework of the Interreg Med Plastic Busters MPAs project, building on the most recent methodological advances of the MSFD TGML, INDICIT II Project and Barcelona Convention CORMON, and on the results of the project's testing phase.

PREPARED BY

THE INTERREG MED PLASTIC BUSTERS MPAs PROJECT

12.1 Sampling approaches

Fish species should be sampled following one of the following approaches depending on the type of analysis to be performed:

- For the analysis of litter and associated contaminants, fish species (dead) can be purchased by local fishers active in the study area.
- For the analysis of litter, associated contaminants and biomarkers, fish species (live) should be collected in the study area via a dedicated sampling campaign.

It is recommended to record the following information for each sampling site:

- **Climate variables**: Sea temperature (in ºC), mean wave height, maximum wave height, mean wave period, wave direction, etc.
- **Environmental variables:** Sediment granulometry, nutrients, turbidity, chlorophyll-a, salinity, etc.
- **Habitat Characteristics:** Habitat type (e.g., sand, seagrass, algae, mats), habitat composition (% seagrass, % sand), etc.
- **Coastline morphology:** Beach, cliffs, estuaries, closed bay, open bay, creeks, etc.
- **Anthropogenic variables:** Anchoring allowance, diving, sewage input, fishing activities, presence of fishing gear, poaching, etc.
- **Protection status:** Protection level (fully protected, partially protected, not protected), protection status (e.g., Marine Reserve, Natural Park, Site of Community Importance), number of years before/after the establishment of protection status, etc.

12.2 Frequency and timing of surveys

Frequency of sampling is at least once per year, taking into account seasonality.

12.3 Sample size

A minimum of 30 individuals per fish species should be sampled at each site, preferably for each environmental compartment (i.e., benthic, demersal, pelagic). Specimens of endangered species (e.g. Manta ray) occasionally found stranded can also be analyzed in very small numbers.

12.4 Tissues collection

To perform litter, contaminants and biomarker analyses, tissues should be removed from living organisms. Alternatively, if performing only litter and contaminant analysis, tissues can be removed from animals frozen at -20 °C. Before the dissection, the following information should be recorded for each fish sample:

- Date and time of capture
- Name of sampling location
- Name of the boat(s) providing the samples
- Sampling gear
- Latitude and longitude of each point where species are captured
- Sampling depth
- Sample size: number of individuals sampled.

Immediately after sampling, rinse the fish and label the fish samples with a unique ID for each individual.

Before the dissection of the fish:

- Record the name of the species
- Weigh the whole fish
- Measure the total length of the fish
- Record any visible deformations
- Record the gender (if possible)
- Record the maturity stage

To avoid airborne contamination, it is recommended to dissect the specimen and take tissue samples in the laboratory, under controlled conditions.

Dead organisms

Before dissection, thaw fish in the laboratory (if previously stored at -20 °C) at room temperature.

Collect the following tissues:

- Gastrointestinal tract (GI) for litter analysis: whole GI in aluminium foil at -20 °C.
- Muscle for contaminants analysis: about 1g in aluminium foil stored at -20 °C.
- Liver for contaminant analysis: about 1g in aluminium foil stored at -20 °C. Weight the liver for somatic liver index (SLI) evaluation.

Each tissue stored in aluminium foil must be labelled with a unique ID for each individual.

Live organisms

Keep the sampled live animals on board, in seawater with oxygenators, transport and dissect the animals in the laboratory. Alternatively, animals can be dissected on board. Before dissection, anaesthetise the animals following related guidelines.

Extract the following tissues:

- Blood samples for biomarker analysis: the blood should be withdrawn from the caudal vein using a disposable heparinized syringe. Use part of the blood to obtain blood smears and centrifuge an aliquot of the blood to obtain plasma samples.
- Liver for biomarker analysis: about 1g in aluminum paper, weight the liver for somatic liver index (SLI) evaluation, freeze in liquid nitrogen or dry ice in cryovials and store at -80°C.
- Kidney for biomarker analysis: frozen in liquid nitrogen or dry ice in cryovials and stored at -80°C.
- Gills for biomarker analysis: frozen in liquid nitrogen in cryovials or dry ice and stored at -80°C.
- Muscle sample: an aliquot frozen in liquid nitrogen or dry ice in cryovials and stored at -80 °C for biomarker analysis, and a part of the sample for contaminants analysis in aluminium foil, stored at -20 °C.
- Gastrointestinal tract (GI): whole GI in aluminum foil stored at -20°C for litter analysis.

Each tissue stored in aluminium foil or cryovial/eppendorf must be labelled with a unique ID for each animal.

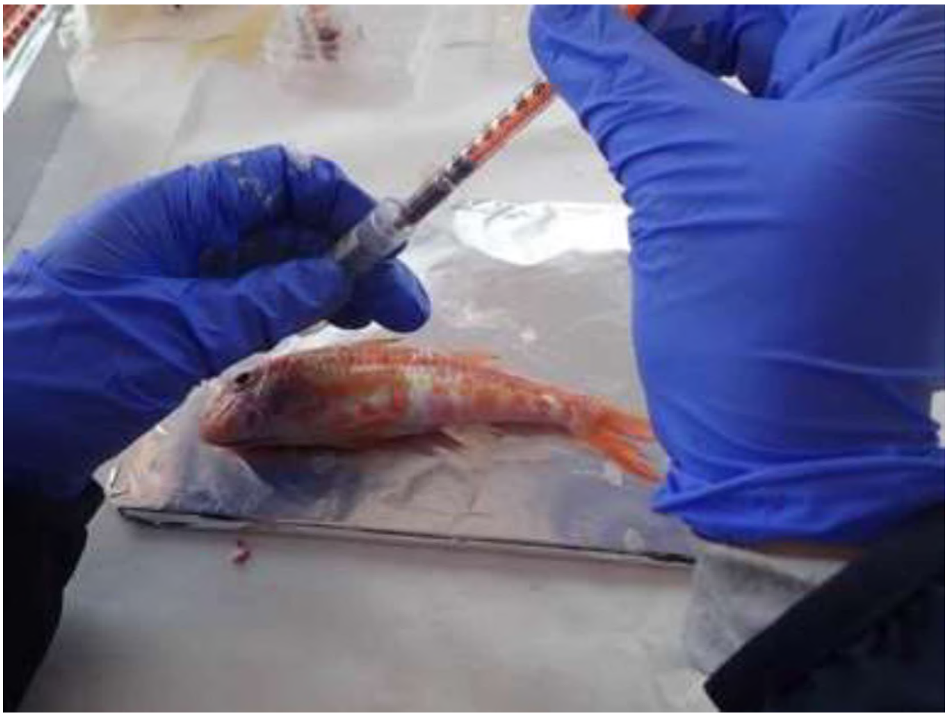


Figure 12-1. *Fish blood sampling (live fish).*

12.5 Litter size classes to be surveyed

Litter items smaller than 5 mm can be classified in different size classes, large and small microplastics. Lowest limit for microplastic should be 100 µm. For large fish species (e.g. swordfish, tuna), where is possible, under notes in datasheets, the items should be described and assigned a litter category number using the "Joint List" developed by the MSFD TGML group (Fleet et al., 2021).

12.6 Litter analysis and classification

- Place GI (stomach and intestine) in a glass petri dish or beaker. Be careful to annotate the fish ID in each petri-dish/beaker.
- Weigh and rinse the GI with purified water (e.g. milli Q).
- Place a filter paper in a petri dish (blank sample) in the working area during fish dissection to test airborne contamination.

Macrolitter detection

- For macrolitter and gut content analysis, cut open the stomach and intestine, remove stomach and intestine contents and weigh separately.
- Sort prey or litter items into separate categories under a stereomicroscope, taking care of recording their weight.
- Measure the size of litter items and classify litter categories classified according to the JointList of Litter Categories of the MSFD Technical Group on Marine Litter.

In addition, the following parameters should be recorded:

- Record for all categories (litter and other elements) the dry mass (grams, precision 0.01 g) of each category: dry the sample at room temperature for 24 h minimum or in a stove at 35 °C for 12 h.

Microlitter detection

For microlitter analysis apply the following digestion procedure described in Tsangaris et al. (2021), which is the result of an intercalibration among the PB-MPAs project partners. To avoid losing content, digest the entire GI and not just its content. The GI can be divided in two subsamples for faster digestion since time required for digestion depends on the amount of tissue to be digested.

Microplastic and macroplastic analysis

For microplastic and microplastic analyses apply the following procedure:

- Place the entire GI in a glass beaker, add 5ml 10% KOH per gram of tissue wet weight (1:5 w/v).
- Cover the samples with aluminum foil and heate on thermostatic water bath (50 °C) until all organic matter is removed (maximum 2 days, 12 hours heating).
- After the digestion of the organic matter, pass the samples through a metal sieve (300 μm) placed above a filtering apparatus and finally filter under vacuum onto a fiberglass filter (Whatman GF/C, pore size 1.2 or 1.6 μm).
- Metal sieves should be covered with aluminum foil and filters must be placed in aluminum foil-covered Petri dishes and dried at room temperature.
- Use a procedural blank sample to test for possible ambient contamination: add similar volume of 10% KOH as that used in the samples in a beaker without sample, and follow the protocol described in the steps above.
- After the digestion procedure, check the filter for plastic items with the use of a stereomicroscope.
- Photograph, count and record the type, colour and maximum length of plastic particles using image analysis software. Categorize plastic particles according to shape, size, colour and polymer.
- The recovery rate of microplastics by the applied extraction procedure must be tested on tissue samples enriched with specific number (e.g. 10 particles/sample) of different types of plastic particles. The number of particles detected after sample processing is used to calculate % recovery rate of microplastics.
- To avoid contamination, carry out filtration under a cover (e.g. glove bag, laminar flow cabinet or other closed cover).
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.
- Test recovery of microplastics by the applied extraction procedure on fish tissue samples enriched with specific number (e.g. 10 particles/sample) of different plastic particles of known polymer type and size (positive controls, minimum number: 3). The number of particles detected after the processing of these samples as described above, should be used to calculate % recovery of microplastics.

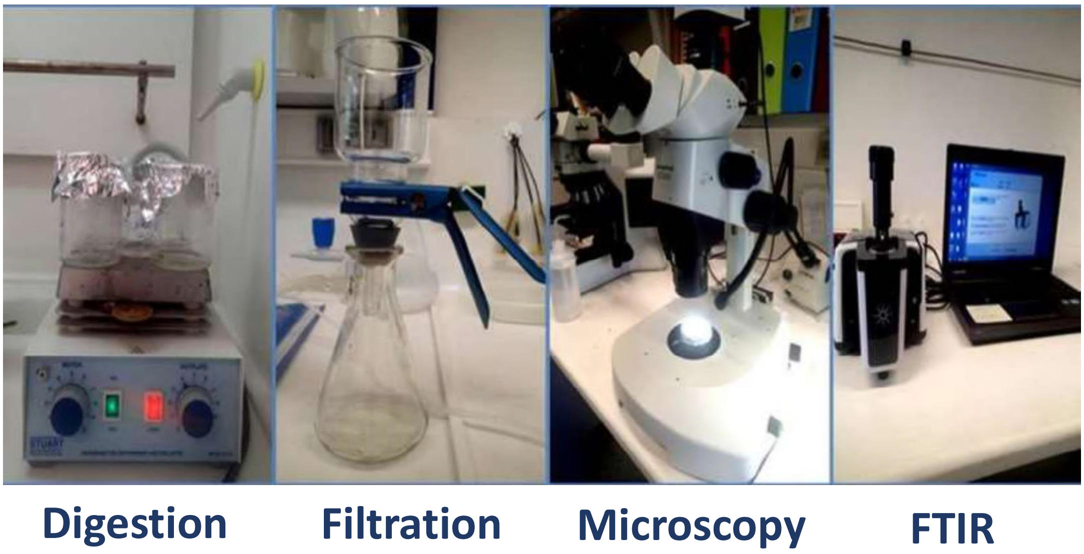


Figure 12-2. *Main steps of fish sample processing for microplastic detection (from Tsangaris et al. 2015).*

Contamination precautions

Contamination precautions are essential during all steps of the sample processing due to the ubiquitous nature of certain types of microplastics, such as synthetic fibers, that can contaminate the samples. Glass material should be used where possible and all glassware and tools (e.g. tweezers, scissors, etc.) should be rinsed thoroughly with purified water. Staff should wear natural fiber laboratory clothes. Sample processing should be done in closed areas with little ventilation and air circulation for example from air conditioners. Samples should be covered by foil paper during digestion and when not in use. It is recommended to use covers during sample rinsing and filtration (e.g. glove bag, laminar flow cabinet or other closed cover) and during all steps of samples processing (e.g. dissection, examination under the stereomicroscope). Procedural blank samples should be throughout the entire sample processing. During the analysis's procedure, two glass petri dishes should be placed at each side of the stereomicroscope and checked for microplastics before and after each sample. A 100% cotton laboratory coat shall be worn at all times during the procedure.

Figure 12-3. *Filtration of digested sample in a glove box.*

Collection of data

For each organism an assessment is made of the:

3. Frequency of occurrence (%) of ingested macro- and microplastics for each species is calculated as the percentage of the individuals examined with ingested microplastics.
4. Abundance (N) of macro- and micro-plastics ingested per individual (average number of items/individual) for each species is calculated as a total and per category. Since currently there are inconsistencies in the literature in reporting abundance of ingested litter, it is recommended to report average number of items per individual both considering all individuals examined and only individuals found with ingested macrolitter and microlitter.
5. The percentage of the individuals affected in relation with the individuals of the whole sample examined (all species).

For each organism data on litter ingested is reported:

1. Characteristics of the litter found (colour, shape, size, polymer) in each specimen according to the “MSFD Protocol for the monitoring of microliter ingested by marine fish”.
2. The number, length, weight and nature of the polymer (10%) of the items examined for each species.
3. Recovery rate of microplastics.

12.7 Analysis of plastic tracers and PBTs

Plastic additives

The compounds to be detected in different tissues/fluid are:

- *Phthalates:* a group of chemicals widely used as additives to make plastics more flexible and harder to break; they can interfere with endocrine system (Baini et al., 2017).
- *Bisphenol A*: used in the production of polycarbonate, can have endocrine disrupting effects (Crain et al., 2007; Halden, 2010; Oehlmann et al., 2009) and the styrene and polyvinyl chloride monomer, used in the production of polystyrene and polyvinyl chloride (PVC), can be carcinogenic and/or mutagenic (Lithner et al., 2011; Papaleo et al., 2011; Xu et al., 2004).
- *Polybrominated diphenyl ethers:* they belong to the group of brominated flame retardants (BFRs), which are used in various polymeric materials such as plastic parts, resins, textiles, and other substrates to reduce their fire hazards (BSEF 2003; Król et al. 2012).

Persistent, bioaccumulative and toxic substances (PBTs)

In addition to the plastic additives that may leach from plastics when released into the marine environment, plastics tend also to adsorb in their surface persistent bioaccumulative and toxic substances (PBTs) (e.g. organochlorine compounds OCs, PAHs and PBDEs) and metals (e.g., lead, copper and cadmium) that are present in the seawater.

Depending on the compounds and the tissue to be analysed, different methods should be applied to detect the presence of plastic-related contaminants in the fish species (Annex III).

12.8 Biomarkers analysis

The toxicological effects associated with the presence of marine litter can be evaluated using a set of diagnostic and prognostic methodologies, by means of biomarkers. A non-exhaustive list of existing biomarker approaches and plastic tracers' contaminants that are usually applied in fish analyses is reported in Annex III.

Biomarkers have been selected on the basis of the level of biological responses and in relation to the main effects related to marine litter/microplastics ingestion. The selected biomarkers can diagnose different impacts related to: a) physical damages/effects of marine litter, b) exposure to/effect of chemical tracers, and c) exposure to/effect of adsorbed chemicals.

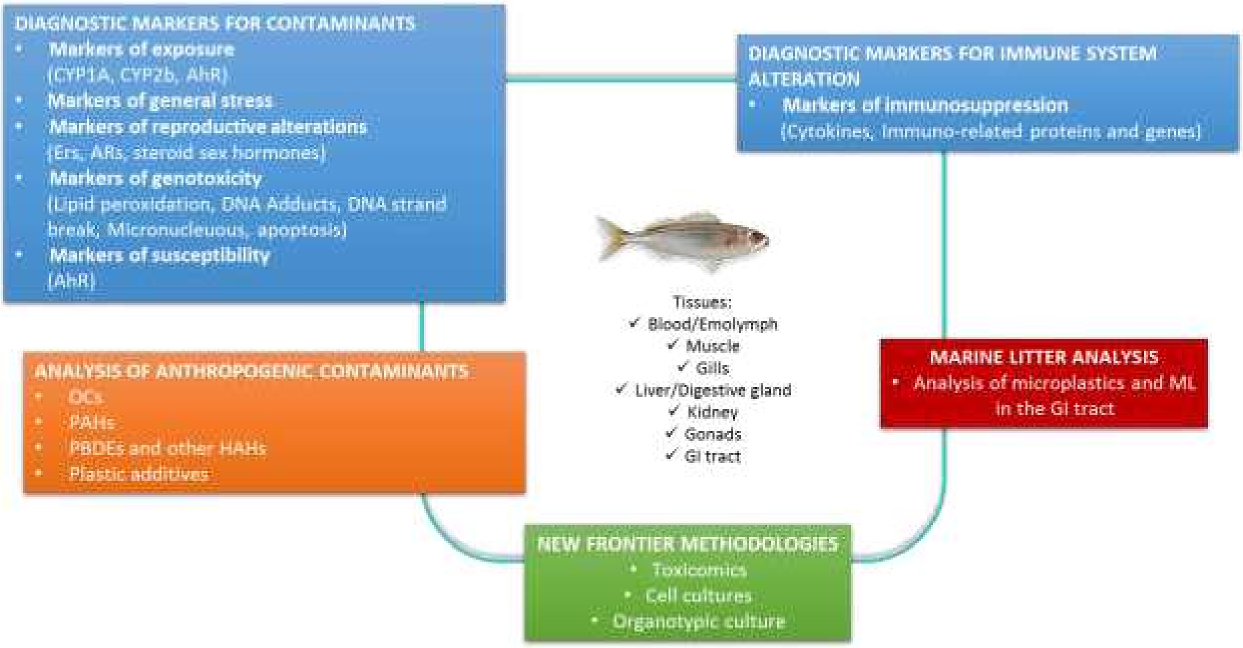
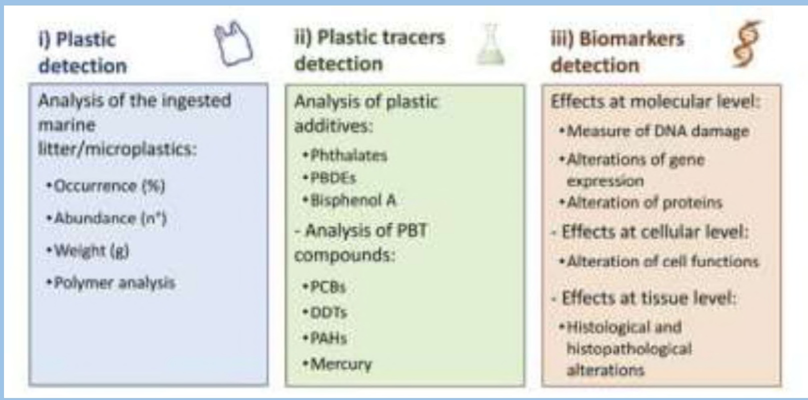


Figure 12-4. *A three-fold approach to detect the marine litter presence and impacts to fish species.*

Starting from this initial list and building on the findings of the testing phase of the Plastic Busters MPAs project, the most suitable diagnostic tools to detect the presence and impact of marine litter on fish are proposed here below.

Table 12-1. *Main diagnostic tools selected in the Plastic Busters MPAs project to detect the presence and impact of ML in fish.*

FISH: MAIN DIAGNOSTIC TOOLS SELECTED IN THE PLASTIC BUSTERS MPAs PROJECT



1. Analysis of gastrointestinal content: Litter analysis and classification
2. Analysis of plastic tracers: Phthalates
3. Analysis of biological end-points: ENA assay, LPO, AChE, gene expression (*elovl6, traf3, cyp3a, ifitm1, msmo1, nf-kb, pparg, ppara, gpx)*

12.9 Materials & Equipment

Material for sampling

- Camera
- Containers for samples, zipped bags, cool boxes
- Garbage bag
- Gloves
- Disposable scalpels, fine forceps, scissors and tweezers for dissection
- Dissection board
- Measuring decimetre
- Pen/pencil/ Permanent marker
- Sampling sheets
- Aluminium foil
- Cryoboxes
- Cryovials
- Liquid nitrogen Dewar (in alternate dry ice)
- Paper and block-notes
- Paper towels

Material for macroplastics and microplastics analysis

- Distilled water
- Permanent marker
- 10% KOH
- Disposable scalpels, fine forceps, scissors and tweezers for dissection
- Precision tweezers (fine and pointed) for micro-plastic handling on filters and FTIR

- Petri dishes
- GFC filters 0.2, 1.2 or 1.6 µm 47 mm diameter for filtration
- Aluminum foil
- 150 and 250 ml glass beakers
- 100 ml measuring glass cylinder
- Magnetic stirrer
- Hot plate
- Glove bag, laminar flow cabinet or other closed cover
- Vacuum filtration system
- Analytical balance
- Stereo microscope with image analysis software
- FTIR or Raman spectroscopy with associated analysis software

References

Guidance on Monitoring of Marine Litter in European Seas, 2022. A guidance document within the Common Implementation Strategy for the marine Strategy Framework Directive - Marine Litter Impact on Biota. MSFD Technical Group on Marine Litter - draft.

INDICIT II, Final report, 2021. Deliverable D1.6, of the European project "Implementation of the indicator of marine litter on sea turtles and biota in Regional Sea conventions and Marine Strategy Framework Directive areas".

Fossi, M.C, Vlachogianni, T., Anastasopoulou, A., Alomar, C., Baini, M., Caliani, I., Campani, T., Casini, S., Consoli, P., Cillari T., D'Alessandro, M., Deudero, S., Galgani, Galli M., F., Kaberi H., Panti, C., Pedà, C., E. Romeo, T., Scotti, G., Tsangaris, C., Zeri, C., 2019. Toolkit for monitoring marine litter and its impacts on biodiversity in Mediterranean MPAs. Interreg Med Plastic Busters MPAs project (D.3.3.2).

12.10 Sampling & recording sheets

Monitoring Marine Litter (Macro-Micro) in biota: dead fish

Sampling date and time	Sampling site	Boat name	GSA	Sampling gear	Depth	Coordinates	
						Latitude	Longitude

ID code	Species	Sex	Total length (cm)	Total weight (g)	GI weight (g)	Muscle	Liver weight (g)

Notes and remarks:

Rack (N_2)

Monitoring Marine Litter (Macro-Micro) in biota: live fish

Sampling date and time	Sampling location	Boat name	GSA	Sampling gear	Depth	Coordinates	
						Latitude	Longitude

ID code	Species	Sex	Total length (cm)	Fork length (cm)	Total weight (g)	GI weight (g)	Muscle	Liver weight (g)	N°. of liver aliq.	Bile	Brain	Kidney	Gonad weight (g)	Blood smears	Plasma

Notes and remarks:

Rack (N_2)

ANNEX III

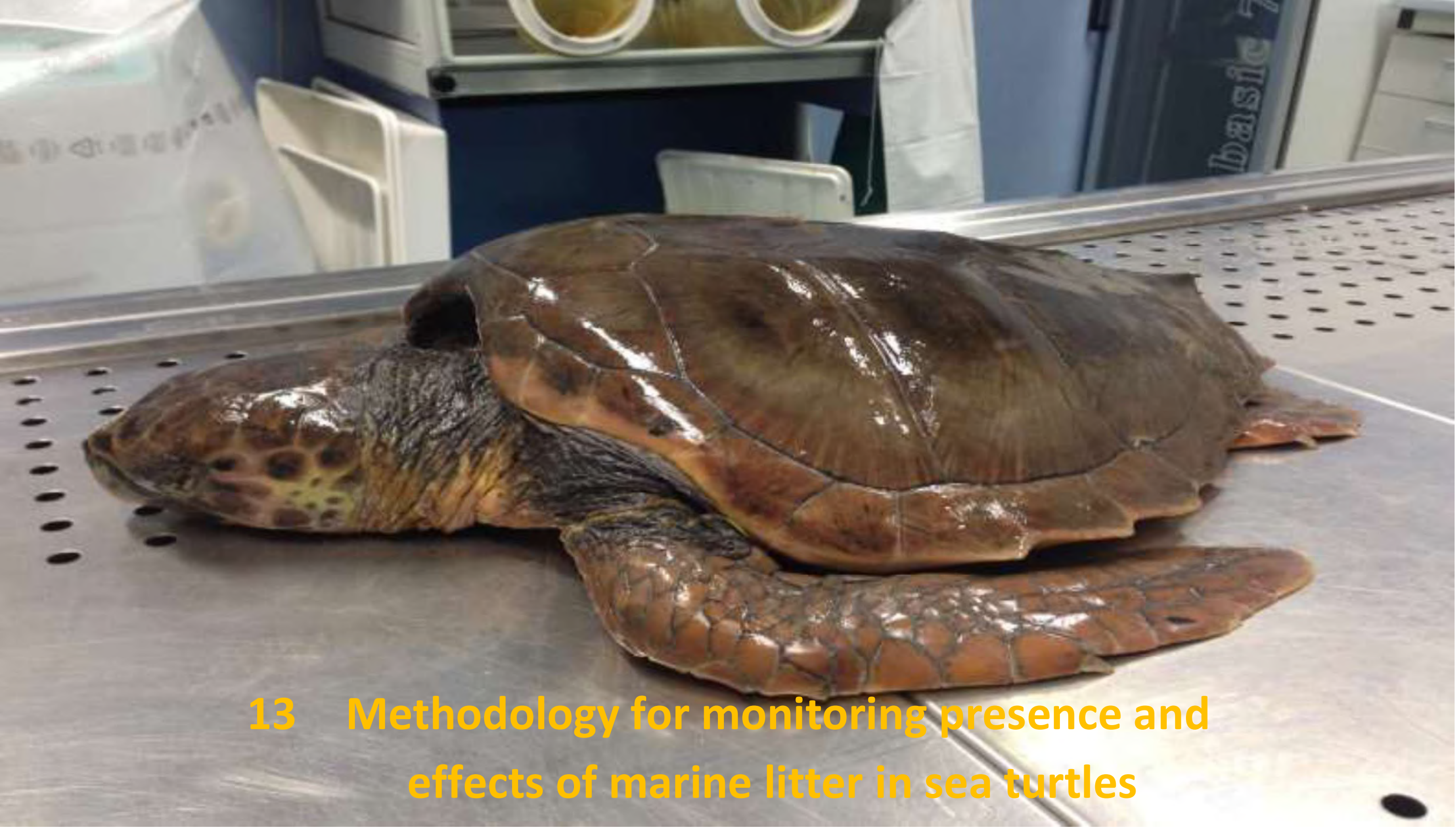
Table A-3. *Tissues and methods to be used to detect plastic tracers in fish.*

	CHEMICAL COMPOUND	TISSUE/SAMPLE	ANALYTICAL METHOD
PLASTIC ADDITIVES	Phthalates	muscle, liver, whole organism	Baini et al., (2017), Fossi et al., (2016), Savoca et al., (2018)
		Blood	Takatori et al., (2004)
	Bisphenol A	Muscle	Ballesteros-Gómez et al., (2009), Barboza et al., (2020)
		Blood	Cobellis et al., (2009)
	Polybrominated diphenyl ethers	Muscle, liver, blood	Muñoz-Arnanz et al., (2016), Bartalini et al., (2019), Ameur et al., (2020), Corsolini et al., (2008)
ADSORBED CONTAMINANTS	**Polycyclic aromatic hydrocarbons**	Muscle, liver, blood	Marsili et al., (2001), Frapiccini et al., (2020)
	Organochlorine contaminants	Muscle, liver, blood	Marsili and Focardi, (1997), Bartalini et al., (2019), Garcia-Garin et al., (2020)
	Mercury	Blood, muscle	Correa et al., (2013), Barboza et al., (2018)

Table A-4. *Effects measured in fish by the biomarker approach.*

EFFECT	TISSUE	TEST
GENOTOXICITY	Blood	Comet assay (Molino et al., 2019) (*) Mn test (Bolognesi et al., 2006) ENA assay (Pedà et al., 2022); (Pacheco and Santos, 1997)
OXIDATIVE STRESS	Liver, kidney, gill	CAT, GST, LPO, GPX, GR, GSH (Pedà et al., 2022 ; Yu et al., 2018) (*) qPCR NRF2, CAT, SOD (Espinosa et al., 2019)
	Plasma	LPO (Pedà et al., 2022 ; Campani et al., 2020, Casini et al., 2018) CAT (Pedà et al., 2022)
IMMUNOTOXICITY	Blood, liver	Total and differential white blood cells (WBC) count (Casal and Orós, 2007; Davis et al., 2008; Caliani et al., 2019) H:L ratio (Caliani et al., 2019) Respiratory burst (Secombes, 1990; Caliani et al., 2019) TAS assay (Miller et al., 1993; Caliani et al., 2019) Lisozyme enzyme (Keller et al., 2006; Caliani et al., 2019) (*) Transcriptomics (Limonta et al., 2019) qPCR IL1B, IL8 CASP3 (Espinosa et al., 2019); CASP8, CASP9, TRAF (Karami et al. 2017)
REPRODUCTION	Plasma, Gonads, Liver	CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015) (*) Vitellogenin (Fossi et al., 2004), Vtg (Mak et al., 2019)
	Plasma	Vitellogenin (Herbst et al., 2003) CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*)
HISTOPATHOLOGY INFLAMMATION AND MORPHOLOGY	Liver, kidney, gill	Histopathology,histology (Pedà et al. 2016; Karami et al. 2017; Batel et al., 2018) (*)
XENOBIOTIC METABOLISM AND BIOTRANSFORMATION	Liver, blood, bile	Porphyrins (Grandchamp et al. 1980; Guerranti et al. 2014) (*) Bile metabolites (Oliveira et al 2013) (*) EROD (Zhang et al., 2019) (*)
	Blood, skin, liver	CYP1A; AHR, CYP3A (Fossi et al. 2014, Panti et al. 2011; Rochman et al., 2013) (*) Porphyrins (Guerranti et al., 2014) (*)
NEUROTOXICITY	Brain, muscle, plasma	AChE, BChE (Barboza et al., 2018) (*)
CELLULAR STRESS	Whole organisms, muscle	Lysosomal membrane stability-LMS (Canesi et al 2015) (*) IDH (Oliveira et al., 2013) (*)
	Blood, skin, liver, kidney	PPARA, PPARG, HSP70, GPX, E2F1 (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*) Gamma glutamyl transferase (GGT) (Nematdoost Haghi and Banaee, 2017) (*) Cortisol and corticosterone (Flower et al., 2015) LDH (Nematdoost Haghi and Banaee, 2017) (*)

(*) effects detected after laboratory or field exposure with MPs or Plastic-related contaminants.



13 Methodology for monitoring presence and effects of marine litter in sea turtles

This document describes the methodological approach for monitoring the presence and effects of marine litter in sea turtles, which has been developed within the framework of the Interreg Med Plastic Busters MPAs project, building on the most recent methodological advances of the MSFD TGML, INDICIT II Project and Barcelona Convention CORMON, and on the results of the project's testing phase.

PREPARED BY

THE INTERREG MED PLASTIC BUSTERS MPAs PROJECT

Interreg Mediterranean

PLASTIC BUSTERS MPAs

13.1 Species sampling

The presence and impact of marine litter in Mediterranean sea turtles (*Caretta caretta, Chelonia mydas* and *Dermochelys coriacea*) can be investigated and/or monitored in:

- Dead organisms that may have been stranded ashore, captured by fishers (by-catch), found at sea or died at a recovery centre.
- Living organisms that have been hospitalized in a rescue centre.

13.2 Description of investigated turtle and biometric measurements

Description of the investigated turtle

Identify the species of the observed marine turtle and in case of doubt about the species identification, refer to an identification guide (e.g. www.cites.org). If the species can't be identified, note it as non-identified (NI) on the observation sheet.

The sea turtles are protected species, therefore only authorized people can handle live and dead animals or parts of them. Upon finding the animal, its management and recovery should be reported and coordinated with the responsible National Authorities. Note that a CITES permit is asked if a specimen or sample has to be sent/received or to be transported.

Indicate the presence and code number of tag, if present, otherwise, note "NO".

Note the date of discovery (dd/mm/yyyy), the location of discovery and the coordinates if available (X, Y: in decimal degrees, or specify the coordinate system); the name and contacts (phone, mail) and institution of the person in charge of the recovery shall also be noted.

Take a photo of the animal before handling.

All the sea turtle's data should be noted down in a sampling sheet (see paragraph 12.9).

Biometric Measurements

Morphometric measurements should be collected before the necropsy (if the animal id dead) or tissue collection (for specimens recovered in a rescue centre). Standard Curved Carapace Length (CCL), notch to tip is mandatory, while other measurements are optional (in centimetres, precision 0.01 cm) in addition to this measures weight (kilograms, precision 0.01 g) and sex of the specimen should be recorded:

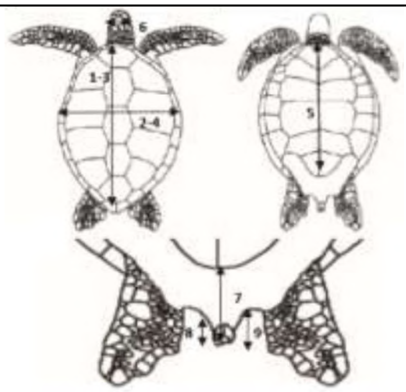
Biometric measurement				
1. CCLst	cm	6. HW	cm	
2. CCWst	cm	7. PTL	cm	
3. SCLst	cm	8. CTL	cm	
4. SCWst	cm	9. CaCL	cm	
5. CPL	cm			
Weight (kg)				
Sex				

Figure 13-1. *Biometric parameters to be measured mandatory (Standard Curved Carapace Length – CCLst, weight and sex) and optional (Standard Curved Carapace Width – CCWst; Standard Straight Carapace Length – SCLst; Straight Carapace Width – SCWst; Curved Plastron Length – CPL; Head Width – HW; Plastron Tail Length – PTL; Cloaca Tail Length – CTL; Carapace Cloaca Lenght - CaCL) (from INDICIT, 2018).*

13.3 Conservation/health status of the organism

- With regards to the status of the organism, two cases are possible: the turtle may be live, or dead. In the case of live animals (Level 1) biological samples (blood, carapace, plasma, biopsy, faeces) can be collected for biomarker and chemical analyses. In case of dead animals, 4 different situations can be observed: in animals that have just died (< 2 hours post mortem), gastrointestinal (GI) system is adequate for litter ingestion analysis, and other tissues (muscle, liver) can be used for biomarker and chemical analyses.
- Levels 2 (fresh), 3 (partial decomposed) and 4 (advanced decomposed): are adequate litter ingestion analysis (in GI) from necropsies and chemical analysis.
- Level 5 (mummified): the litter ingestion analysis is not possible because the individuals have usually lost their gastro-intestinal material.

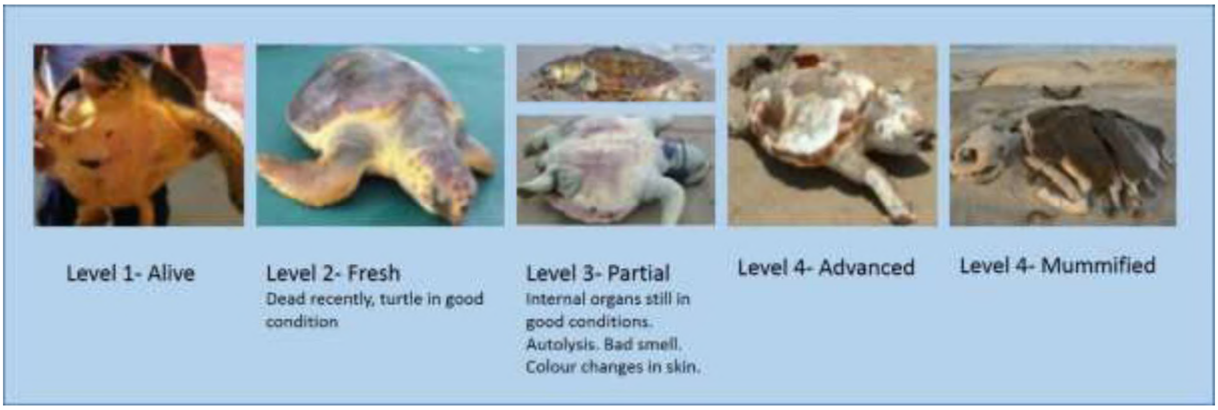


Figure 13-2. *Conservation status codes for stranded organisms (from UNEP/MAP, 2019).*

Discovery circumstances

Note the circumstances among the different categories:

- Stranded: animal found stranded on the beach or in the shoreline.
- By-catch/fisheries: animal accidentally captured by fishers.
- At sea: dead animal found on sea surface.
- Dead RC: the animal arrived live but died during its hospitalization in the rescue centre.
- Unknown = unknown

Possible cause of morbidity and mortality, type of impact

If possible, the type of interaction with human activities and impact observed or suspected on dead or live stranded individuals should be deduced from external or organs observations during the necropsy and complemented with veterinarian examinations.

Also, an inspection of the oral cavity should be conducted for the presence of foreign material. Then one of the following 9 categories should be selected and noted; the remarks box should be completed with the help of the pathologist (if this is requested):

1. Bycatch/Fisheries related: ingested hook, decompression sickness, individual trapped in a fishing gear, individual drowned in a fishing gear;
2. Entanglement in debris: entanglement in litter other than related to fishing activity. Please fill the column "Entanglement type" and "Litter causing entanglement";
3. Ingestion of litter: digestive obstruction or occlusion, perforation or other impacts;
4. Anthropogenic trauma: Collision with a boat or a propeller, individual beaten with knife, stick or harpoon, poaching;

5. Natural predation: usually shark attack;
6. Natural disease: buoyancy trouble, cachexia, dermatitis, conjunctivitis, rhinitis;
7. Oils: Ingestion or external impregnation with oils;
8. Unidentified: Impossible to know the cause of death/stranding, no remarkable damages, injury or disease.
9. Other: Please specify in the column "Notes".

Main injuries

In case of injuries, the ***main type of injury*** (bone fracture, amputation, slightly or deep cuts, throttle, abrasion or other) and the ***affected body part*** should be reported. If the individual has been found entangled in litter, the type of material in which the sea turtle was found should be specified, according to the following categories: *Pieces of net (N), Monofilament line (nylon) (L), Rope or pile of ropes (R), Plastic bag (Pb), Raffia (Rf), Other plastics (Ot), Multiple materials (Mu), Unknown (Unk).*

Health status of live animals

Note the health status according to the level of body condition by visual observation of the plastron shape.

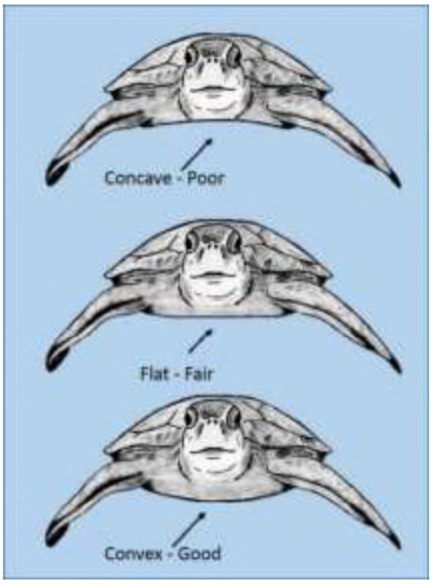


Figure 13-3. *Health status from visual observation of plastron shape: concave plastron poor health status; flat plastron fair health status; convex plastron good health status (from Thomson et al., 2009).*

Other descriptive parameters

Visual inspection of the animal's fat reserves at the neck is recommended. For dead individuals, this can be verified when opening the plastron according to the quantity of fat recovering the abdominal muscles. Choose among the 3 categories:

- Thin (sunken neck)
- Fat
- Normal

If possible, the sex (male or female) should be noted, which can be determined by gonads analysis or, in adult individuals, from the observation of secondary sexual characters (e.g. length of the tail and of the claw in the front flipper). Otherwise, specify by NI (for Not Identified).

13.4 Protocol for dead sea turtles

Necroscopy

The carcass should be placed on its back, trying to wedge it with an object so that it does not wobble from side to side. The plastron should be removed and separated from the carapace through an incision on the outside edge (yellow line). The incision should be made with special attention, with the use of a short blade or by cutting with a horizontal tilt to avoid affecting the integrity of the interior organs.

Once the inside of the plastron is accessed, cut the ligament attachment to the pectoral and pelvic girdle to pull back the plastron and reach the muscles and then the internal organs.

Qualitative evaluation of the trophic status of the animal should be made, including the atrophy of pectoral muscles (none, moderate, severe), fat thickness in joint cavities and on coelom membrane (abundant, normal, low or none).

Figure 13-4. *Sequence of turtle necropsy: a) Ventral view of a dead turtle. Yellow line indicates the way to separate the plastron from the rest of the turtle; b) Horizontal cuts to prevent affecting the interior organs; c) Ventral view of the opened turtle (fat reserves (brown) can be observed on the muscles). (From UNEP/MAP 2019).*

Tissue collection

Before sampling the content of the gastrointestinal tract, collect 10 g each of the following tissues for contaminants analysis, which should be wrapped in aluminium paper and kept at -20 °C:

- Muscle
- Liver
- Subcutaneous fat from different body parts
- Kidney
- Carapace scutes

Each tissue stored in aluminium foil must be labelled with the standard identification code of the animal.

In case of turtles dead in rescue centres (max 1-2 h after death), collect:

- Epidermis for analysis of biomarkers and contaminants: take 10-20 g from the neck and forelegs preserved in aluminium foil, store in liquid nitrogen or dry ice.
- Liver for biomarker and contaminant analyses: 10-20 g wrap in aluminium paper and store in liquid nitrogen or dry ice.
- Blood for contaminants: 5-10 ml in tubes and store at -20 °C.

Gut content analysis

Extraction of the gastrointestinal system: expose the gastrointestinal system (GI) by removing the pectoral muscles and the heart of the animal. The blood can be emptied from the abdominal cavity by carefully rolling the turtle onto a side. Clamp the oesophagus proximal to the mouth and clamp the cloaca, the closest to the anal orifice. Remove the entire GI and place it on the examination surface. Isolate the different portions of GI (oesophagus, stomach, intestines) by strangling and cutting between the 2 clamps (see the blue solid lines) the gastro-oesophageal sphincter and the pyloric sphincter.

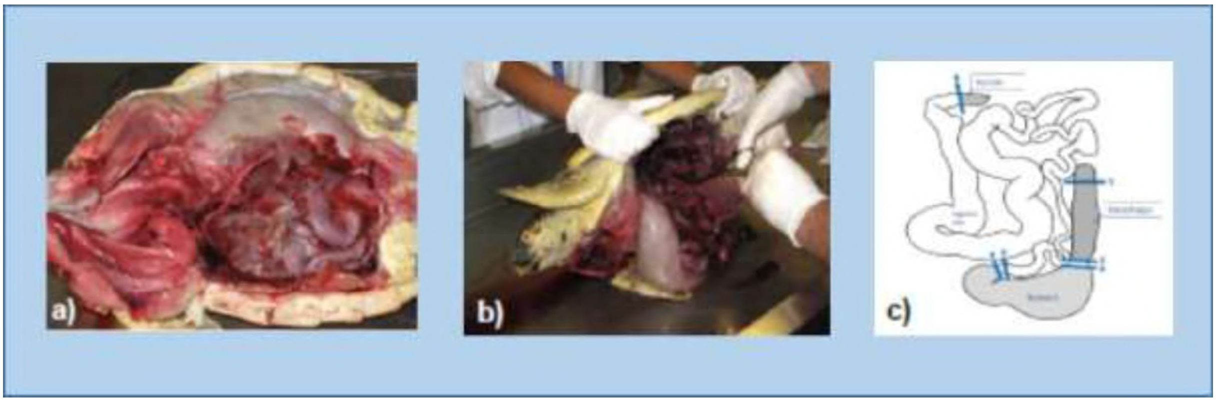


Figure 13-5. *Sequence of extraction and preparation of sections of the digestive tract (GI) a) Remove the pectoral muscle and the heart; b) Extraction of the GI; c) Sketch of the entire GI. Blue lines indicate where clamps must be attached in order to separate the 3 different GI sections (Drawing by V. Hergueta) (From UNEP/MAP 2019).*

Note the external lesions of the GI that can be attributed to litter. Before opening up the digestive tube, examine the outer wall to observe possible perforations by foreign bodies or areas of necrosis. Also note secondary lesions, particularly a peritonitis following on a perforation of the digestive tube, an invagination of the digestive tube, an occlusion, etc. Photograph every lesion observed, taking care to get an overall view as well as close-up (macro-lens) photographs.

The three parts of the gastrointestinal system (i.e. oesophagus, stomach, intestines) should be removed by adding a second strangling at the cut edge to prevent spillage of the content.

Each GI section should be opened lengthways using a scissor and slide the material directly out of the section onto a 1 mm mesh sieve. The content should be cleaned with current and abundant tap water to remove the liquid portion, the mucus and the digested unidentifiable matter.

The content should be inspected for the presence of any tar, oil, or particularly fragile material, which should be subsequently removed and treated separately.

All material should be rinsed, collected in the 1 mm sieve, and placed in tubes or in zipped bags, reporting the sample code (individual code, respective GI section) and stored at -20 °C, pending the laboratory analyses.

For the separation of macrolitter and microlitter, the material should be slid out of the section directly onto a 5 mm mesh sieve superimposed on a 1 mm mesh sieve. Then, proceed with the rinsing and the storing of the material collected as described above, for both 1 and 5 mm sieves, reporting the samples code (individual code, respective GI section and size class (>5mm or 1-5mm)).

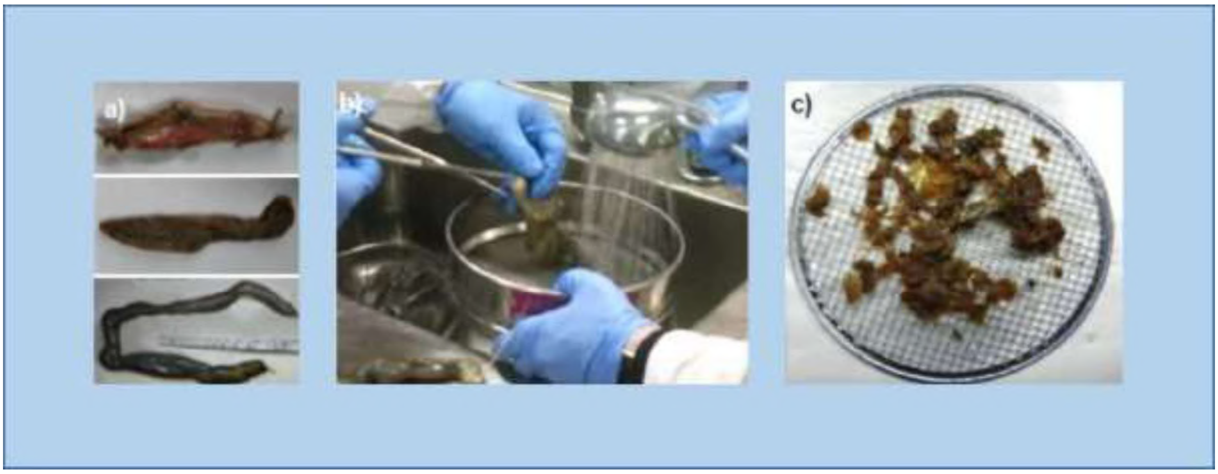


Figure 13-6. *Digestive tract analysis: a) Separated GI sections: Oesofagus (up), stomach (middle) and intestines (down); b) Section opening and gut content lavage; c) Gut content extracted. (From UNEP/MAP 2019).*

13.5 Protocol for live sea turtles

Live sea turtles hospitalized in rescue centres should be manually removed from water for the 30 min sampling period. The cares and procedures carried out on the rescued turtles for all the rehabilitation period should be performed in accordance with routine veterinary practices and guidelines for the conservation and rehabilitation of marine turtles. All the biological samples collected will be used for biomarker and chemical analyses.

Tissue collection

The collection of biological tissues such as blood and skin biopsy must be made with the support of the centres' veterinary while faeces can be collected by the volunteers or the operators of the rescue centre. Each tissue, stored in aluminium foil or Eppendorf, must be labelled with a unique ID for each individual.

Blood sampling

Blood samples (2-6 mL) are to be obtained from the dorsal cervical sinus using a disposable syringe. A small amount should be used for blood smears and the rest transferred into solvent-rinsed glass vials (10 or 5 ml) with Teflon caps containing heparinized saline (heparin sodium) following gently mixing of the tubes.

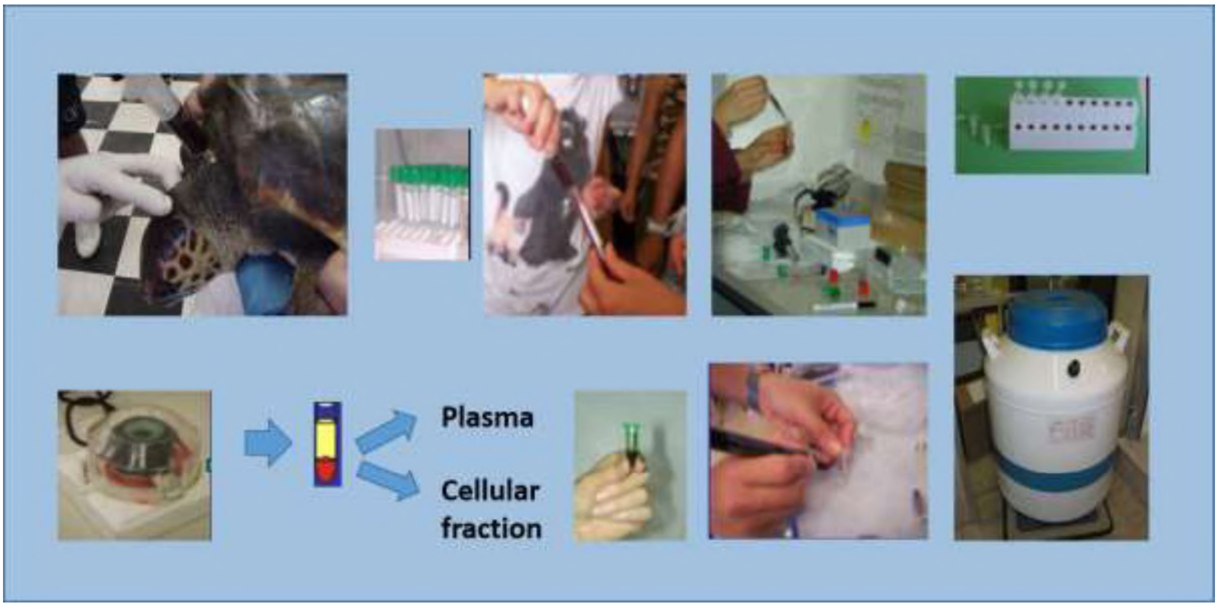


Figure 13-7. *Blood collection and procedure for its processing and conservation.*

A small amount of blood (two drops) is used for each animal to obtain two **blood smears**. Two blood smears for each sample are prepared in double (two slides). Once the blood is taken using a syringe, a drop of blood must be transferred to each slide. The smear of blood is done using a third clean slide as shown in the picture. Allow the blood film to air-dry. Slide fixing should be done the same day of sampling, after the slide is completely dry. Immerse the slides in ethanol for 10 minutes. Allow the blood film to air-dry. Place the slides in the appropriate slide boxes for further analysis.

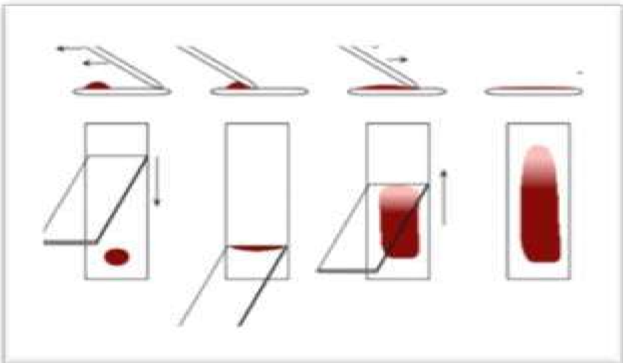


Figure 13-8. *Blood smear technique explained in 4 steps.*

A part of the blood (2 – 5 ml) is transferred into smaller (1.5 ml) centrifuge tubes and centrifuged at 5000 x g for 5 minutes for the separation of **plasma** that is immediately transferred into smaller plastic tubes (0.5 ml) containing a small amount of antiprotease cocktail (5 µl) and stored into dry ice or liquid nitrogen (make a small hole in the upper part of the tubes to avoid the break when taking them out of the liquid nitrogen).

A part of the **whole blood** (1 ml) is stored without centrifugation in plastic tubes in liquid nitrogen or dry ice or -20°C.

500 uL of whole blood and 500 uL of mixture (RPMI and DMSO conservation mixture, 80:20) will be transferred into smaller (1.5 ml) centrifuge tubes and placed into liquid nitrogen or dry ice for biomarker analysis (comet assay).

Skin biopsy sampling

Skin biopsy is performed (eventually after local anaesthesia and disinfection of the skin) using sterile iron punches. The dimension of the punches (diameter: 4 or 6 or 8 mm) depends on the weight of the animal. Once collected, the biopsy must be stored in aluminium foil and stored immediately in liquid nitrogen for enzymatic, cellular and molecular biomarkers. If the liquid nitrogen is not available, the biopsy can be stored in RNA-Later in an Eppendorf at room temperature for 24 hours and after at 4 °C.

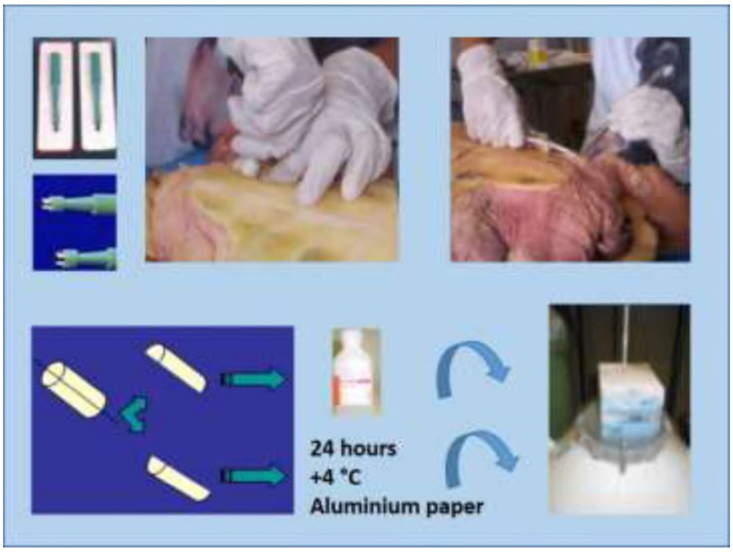


Figure 13-9. *Skin biopsy removal and storage of the samples.*

Carapace sampling

A small amount of the superficial part of the carapace (free of epibionts, barnacles or algae) is to be removed by using a sterile scalpel; from several scutes, approximately 0.25 g of tissue (about 1-2 mm thick) are carefully removed using a disposable scalpel with a plastic handle and a stainless steel blade, by moving parallel to the carapace surface. Only the most superficial keratinous layer must be taken without penetrating the keratinous layer-bone interface below. Scute scrapings taken from carapace will be stored in aluminium paper at - 20 °C.

Figure 13-1. *Carapace collection procedure and storage of the samples.*

Faeces collection

From the day that the animal arrives at the rescue centre, the excreta must be collected every day (or whenever excreta are expelled) for 2 months. The collected faeces will be analysed only for the individuals remaining at least 1-month minimum in the rescue centre. The collection method is as follows:

- Control the water tank daily by filtering through the 1 mm mesh sieve according to the following methods: collect the faces manually with a 1 mm mesh dip net or put a 1 mm mesh flexible collector in the drain tube or place a 1 mm mesh rigid sieve under the drain
- Collect and store excreta and plastic in the same tube
- Use plastic containers (phthalate free) for collection
- Seal the test tube with the code, store the tube at -20 °C

Collect the excreta for the entire period of hospitalization.

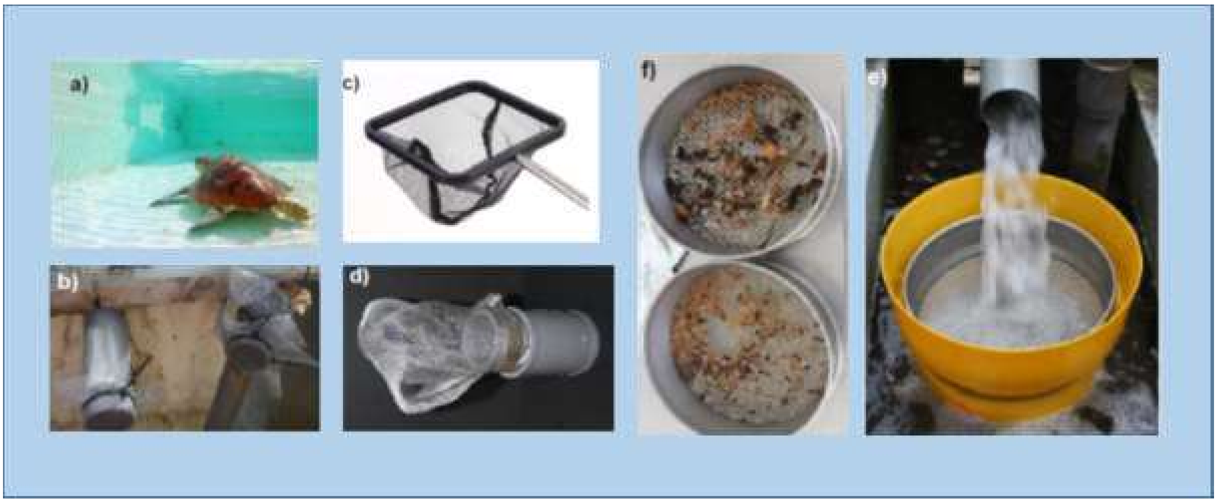


Figure 13-11. *Sequence of faeces sampling. A) The turtle is disposed in an individual tank; b) A 1mm mesh sieve is disposed in discharge tubes; c) A 1 mm dip net for handling faeces; d) Collector with 1 mm mesh disposed in discharge tube for filtering water tank; e) An 1 mm mesh rigid sieve down discharge tube for filtering water tank; f) Sample collected in a rigid sieve (from MAP/UNEP 2019).*

13.6 Litter analysis and classification

Macrolitter detection

- Sort prey and/or litter items into separate categories under a stereomicroscope, taking care of recording their weight.
- Measure the size of litter items and classify litter categories.

In addition, the following parameters should be recorded:

- For all categories: the dry mass (grams, precision 0.01 g) of each category; dry the sample at room temperature for 24h minimum or in a stove at 35 °C for 12 h.
- For litter categories only: the number of fragments and items in each category; a fragment is a piece of litter that can be identified while an item is a set of fragments that seem to originate from the same piece of litter.
- For the plastic litter categories only: the total number of plastic fragments per colour category, with specifics as follow:
  - Total number of white-transparent plastic fragments.
  - Total number of dark coloured plastic fragments (black, blue, dark green…).
  - Total number of light-coloured plastic fragments (cream, yellow, pink, light green…).
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

Microlitter detection

- Examine the fraction 1-5 mm in the Petri dish under a stereomicroscope for particles resembling microplastics. Cover the petri dish with glass lids during observation not to contaminate the sample.
- Photograph, count and record the type, colour and maximum length of microplastic particles using image analysis software.
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

The limit detection for MSFD is 1 mm. Building on the findings of the testing phase of the Plastic Busters MPAs project, it's recommended to also examine the fraction 0.1-1 mm in stranded organisms.

Litter categories

Categorize marine litter according to the categories showed in Table 13-1. The categorization of gastrointestinal tract contents and excreta 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 in most cases, particles can't be unambiguously linked to particular objects. But where is possible, under notes in datasheets, the items should be described and assigned a litter category number using the "Joint List" developed by the TSG ML group (Fleet et al., 2021). In addition, it is important to measure and quantify also natural items (food and/or no food).

Table 13-1. *Classification of Marine Litter items plus Food remain and Natural no food remain (from INDICIT 2018).*

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, e.g. from bag, cling-foil, agricultural sheets, rubbish bags..
Use thread	USE THR	Threadlike materials, e.g. pieces of nylon wire, net-fragments, woven clothing...
Use foam	USE FOA	All foamed plastics e.g. polystyrene foam, foamed soft rubber (as in mattress fillling)..
Use fragment	USE FRAG	Fragments, broken pieces of thicker type plastics, can be a bit flexible, but not like sheet like materials
Other use plastics	USE POTH	Any other plastic type of plastics, including elastics, dense rubber, cigarette filters, balloon pieces, soft airgun bullets... Specify in the column “Notes”.
Litter other than plastic	OTHER	All non-plastic rubbish and pollutant
Natural food	FOO	Natural food for sea turtles (e.g., pieces of crabs, jellyfish, algae...)
Natural no food	NFO	Anything natural, but which cannot be considered as normal nutritious food for sea turtle (stone, wood, pumice, etc.)

Collection of data

For each organism, an assessment is made of:

1. Frequency of occurrence (%) of ingested macro and microlitter for each species, calculated as the percentage of the individuals examined with ingested macro- and microplastics.
2. Abundance (N) of macro and microlitter ingested per individual (average number of items/individual) for each species, calculated as a total and per category. Since currently there are inconsistencies in the literature in reporting abundance of ingested litter, it is recommended to report average number of items per individual considering both all individuals examined and only individuals found with ingested macro and litter.
3. Total dry weight (g) of the detected waste expressed on grams (precision: second decimal place). This weight refers to each single category found in a specific organ (or faeces) of the specimen.

Other information as colour of items, polymer of the different items (at least 10% of the total items) and different incidence of litter in oesophagus, stomach and intestine, incidence and abundance are useful for research and impact analysis.

13.7 Analysis of plastic tracers and PBTs

Plastic additives

The compounds to be detected in different tissues/fluids are:

- *Phthalates:* a group of chemicals widely used as additives to make plastics more flexible and harder to break; they can interfere with endocrine system (Baini et al., 2017).
- *Bisphenol A*: used in the production of polycarbonate, can have endocrine disrupting effects (Crain et al., 2007; Halden, 2010; Oehlmann et al., 2009) and the styrene and polyvinyl chloride monomer, used in the production of polystyrene and polyvinyl chloride (PVC), can be carcinogenic and/or mutagenic (Lithner et al., 2011; Papaleo et al., 2011; Xu et al., 2004).
- *Polybrominated diphenyl ethers:* they belong to the group of brominated flame retardants (BFRs), which are used in various polymeric materials such as plastic parts, resins, textiles, and other substrates to reduce their fire hazards (BSEF 2003; Król et al. 2012).

Persistent, bioaccumulative and toxic substances (PBTs)

In addition to the plastic additives that may leach from plastics when released into the marine environment, plastics also tend to adsorb in their surface persistent bioaccumulative and toxic substances (PBTs) (e.g. organochlorine compounds OCs, PAHs and PBDEs) and metals (e.g., lead, copper and cadmium) that are present in the seawater.

Depending on the compounds and the tissue to be analyzed, different methods should be applied to detect the presence of plastic-related contaminants in the sea turtles (Annex IV).

13.8 Biomarkers analysis

The toxicological effects associated with the presence of marine litter can be evaluated using a set of diagnostic and prognostic methodologies, by means of biomarkers. A non-exhaustive list of existing biomarker approaches and plastic tracers' contaminants that are usually applied in sea turtle analyses is reported in Annex IV.

Biomarkers have been selected on the basis of the level of biological responses and in relation to the main effects related to marine litter/microplastics ingestion. The selected biomarkers can diagnose different impacts related to: a) physical damages/effects of marine litter, b) exposure to/effect of chemical tracers, and c) exposure to/effect of adsorbed chemicals.

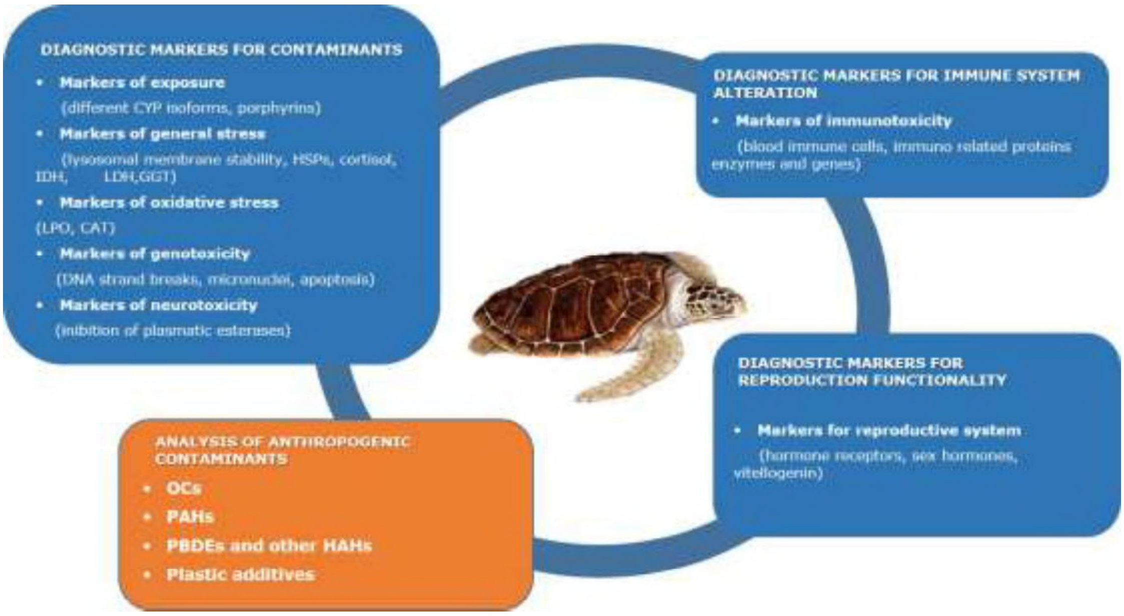
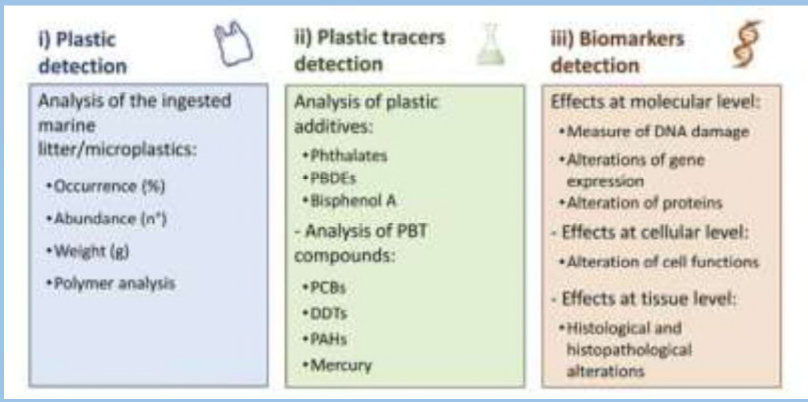


Figure 13-12. *A three-fold approach to detect the marine litter presence and impacts to turtle species.*

Starting from this initial list and building on the findings of the testing phase of the Plastic Busters MPAs project, the most suitable diagnostic tools to detect the presence and impact of ML on sea turtles are proposed here below.

Table 13-2. *Main diagnostic tools selected in the PLASTIC BUSTERS MPAs project to detect the presence and impact of ML in sea turtles.*

SEA TURTLES: MAIN DIAGNOSTIC TOOLS SELECTED IN THE PLASTIC BUSTERS MPAs PROJECT



1. Analysis of gastrointestinal content: litter analysis and classification (dead organisms)
2. Analysis of Faeces: litter analysis and classification
3. Analysis of plastic tracers: phthalates
4. Analysis of biological end-points: ENA assay, porphyrins, lysozyme, gene expression (*cd83, ccr7, lyz, il1b, thra, rxra, ppara, acadl, cyp1a, gst, hsp60, pr, era*)

13.9 Materials & Equipment for sampling

- Boots
- Camera
- Clamps (at least 6) or roast wire
- Clips with claws
- Containers for samples (Bottle/zipped bags)
- Cooler
- Cut-resistant gloves
- Garbage bag
- Glasses and protective mask or shield
- Nitrile Gloves
- Integral protective suit
- Measuring decimetre
- Measuring tape
- Metal containers
- Metal spoon
- Pen
- Permanent marker
- Sampling sheets
- Scalpel
- Scissors
- Sieve with 1 mm mesh

- Sieve with 5 mm mesh
- Transport bins or containers
- Aluminium foils
- Cryoboxes
- Cryovials
- Eppendorf (0.5 ml. 1.5 ml. 2.0 ml)
- Falcon tubes
- Liquid nitrogen Dewar (in alternate dry ice)
- Paper and block-notes
- Paper towels
- Pasteurs
- Pencils
- Plastic Sealable bags
- RNA-Later
- Ruler
- Scalpels
- Thermic bags
- Tweezers

References

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13.10 Sampling & recording sheets

Monitoring Marine Litter in stranded sea turtles

Species:	**ID code:**	
Location/Country:	**Latitude**	**Longitude**

Discovery circumstances	☐ By catch/Fishery ☐ At sea ☐ Stranded ☐ Dead RC ☐ Other ☐ Unknown
Cause of mortality (*Please specify according to the toolkit*)	
Date of discovery	
Date of necroscopy	

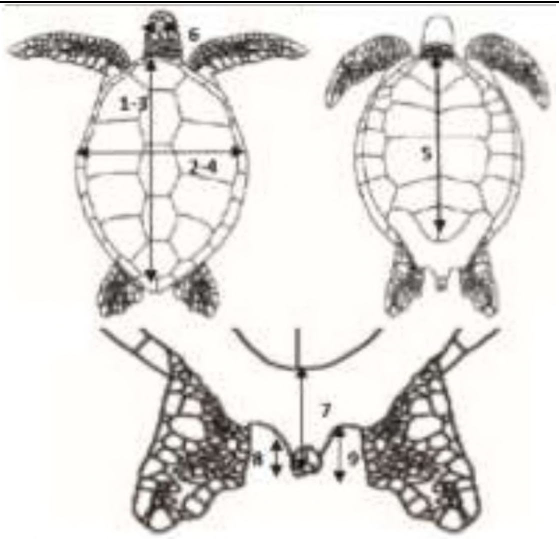
Animal body condition	
Conservation status	☐ Level 1 ☐ Level 2 ☐ Level 3 ☐ Level 4
Healt status (plastron shape)	☐ Poor (concave) ☐ Fair (plane) ☐ Good (convex)
Main injuries	☐ No injuries ☐ Fracture ☐ Amputation ☐ Sectioning ☐ Abrasion
Affected parts	☐ Wings ☐Tail ☐Neck ☐ Head ☐ Other
Fat reserve	☐ Thin ☐ Fat ☐ Normal ☐ Not recorded (NR)

Collected tissues		**N°. of aliquots**
Muscle		
GI tract		
Liver		
Fat tissue		

Picture ☐	**Picture ID:**

Necropsy performed by:

Name and Institution:

<table>
<tr><th colspan="5">Biometric measurement</th></tr>
<tr><td>1. CCLst</td><td>cm</td><td>6. HW</td><td>cm</td><td rowspan="7">
</td></tr>
<tr><td>2. CCWst</td><td>cm</td><td>7. PTL</td><td>cm</td></tr>
<tr><td>3. SCLst</td><td>cm</td><td>8. CTL</td><td>cm</td></tr>
<tr><td>4. SCWst</td><td>cm</td><td>9. CaCL</td><td>cm</td></tr>
<tr><td>5. CPL</td><td>cm</td><td colspan="2"></td></tr>
<tr><td colspan="4">Weight (kg):</td></tr>
<tr><td colspan="4">Sex: ☐ Male ☐ Female ☐ Not identified</td></tr>
</table>

Note and remarks:

Monitoring Marine Litter in live sea turtles

Species:			**ID code:**		
Date and Time: **Arrival**			**Sampling**		
Location/Country:					
Rescue site:		**Latitude**	**Longitude**	**Rescue centre:**	
Health status:					
Cause of morbidity *(Please specify according to the toolkit)*					
Picture ref.:					
Carapace Length		CCL (cm):		CCW (cm):	
Aprox. Age		☐ Adult	☐ Sub-adult	☐ Juvenile	
Weight (kg):		**Sex**: ☐ Male	☐ Female	☐ Not Identified	
Marine Litter		☐ Entanglement ☐ None	☐ Presence in GI tract		
Aliquots		**Liquid N_2**	**RNA later**	**DMSO**	**Cell medium**
	Whole blood				
	Blood smears				
	Plasma				
	DMSO:RPMI conservation mix				
	Excreta				
	Carapace				
	Adipose tissue				
	Skin biopsy				
Slices	**Treatment**	**Dose**	**Time**	**Hour-notes**	

Excreta collection	
Date	**N°. aliquot stored at -20 °C**

Note and remarks:

ANNEX IV

Table A-5. *Tissues and methods to be used to detect plastic tracers in sea turtles.*

	CHEMICAL COMPOUND	TISSUE/SAMPLE	ANALYTICAL METHOD
PLASTIC ADDITIVES	**Phthalates**	Blubber, muscle, liver	Baini et al., (2017), Fossi et al., (2016), Savoca et al., (2018)
		Blood	Takatori et al., (2004), Notardonato et al., (2021)
	Bisphenol A	Muscle	Ballesteros-Gómez et al., (2009),
		Blubber, liver	Xue et al., (2016), Guerranti et al., (2014), Di Renzo et al., (2021)
		Blood	Cobellis et al., (2009)
	Polybrominated diphenyl ethers	Blubber, muscle, liver, blood	Muñoz-Arnanz et al., (2016), Guerranti et al., (2014)
ADSORBED CONTAMINANTS	**Polycyclic aromatic hydrocarbons**	Blubber, muscle, liver, blood	Marsili et al., (2001), Cocci et al., (2018)
	Organochlorine contaminants	Blubber, muscle, liver, kidney, brain, blood	Marsili and Focardi, (1997), Cocci et al., (2018), Gómez-Ramírez et al., (2020)
	Mercury	Blood, skin, muscle, kidney, liver	Correa et al., (2013), Gómez-Ramírez et al., (2020)

Table A-6. *Effects measured in sea turtles by the biomarker approach.*

EFFECT	TISSUE	TEST
GENOTOXICITY	Blood	Comet assay (Molino et al., 2019) (*) Mn test (Bolognesi et al., 2006) ENA assay (Bianchi et al., 2022); (Pacheco and Santos, 1997)
OXIDATIVE STRESS	Plasma, skin	LPO (Fossi et al., 2016), Casini et al., 2018) CAT (Fossi et al., 2013) Cat, gpx, sod (Coccie et al., 2019)
IMMUNOTOXICITY	Blood	Total and differential white blood cells (WBC) count (Casal and Orós, 2007; Davis et al., 2008; Caliani et al., 2019) H:L ratio (Caliani et al., 2019) Respiratory burst (Secombes, 1990; Caliani et al., 2019; Bianchi et al., 2022) TAS assay (Miller et al., 1993; Caliani et al., 2019; Bianchi et al., 2022) Lisozyme enzyme (Keller et al., 2006; Caliani et al., 2019; Bianchi et al., 2022) casp8, casp9, TRAF (Karami et al. 2017; Mathieu-Denoncourt et al., 2015) (*)
REPRODUCTION	Plasma	CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015) (*) Vitellogenin (Fossi et al., 2004)
	Plasma, skin	Vitellogenin (Herbst et al., 2003) CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*) ERa (Cocci et al., 2018)
HISTOPATHOLOGY INFLAMMATION AND MORPHOLOGY	Liver	Histopathology,histology (Pedà et al. 2016; Karami et al. 2017; Batel et al., 2018) (*)
XENOBIOTIC METABOLISM AND BIOTRANSFORMATION	Blood, skin, excreta, liver	CYP1A; AHR, CYP3A (Fossi et al. 2014, Panti et al. 2011; Rochman et al., 2013) (*) Porphyrins (Guerranti et al., 2014) (*) Cyp1a, Cyp1b, gstt1 (Cocci et al 2018, 2019)
NEUROTOXICITY	Brain, muscle, plasma	AChE, BChE (Casini et al., 2018) (*)
CELLULAR STRESS	Blood, skin, liver, kidney	PPARA, PPARG, HSP70, GPX, E2F1 (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*) Gamma glutamyl transferase (GGT) (Nematdoost Haghi and Banaee, 2017) (*) Cortisol and corticosterone (Flower et

		al., 2015) LDH (Nematdoost Haghi andBanaee, 2017) (*) HSP70, HSP90 (Cocci et al 2018)

(*) effects detected after laboratory or field exposure with MPs or plastic-related contaminants.

14 Methodology for monitoring presence and effects of marine litter in seabirds

This document describes the methodological approach for monitoring the presence and effects of Marine Litter in seabirds, which has been developed within the framework of the Interreg Med Plastic Busters MPAs project, building on the most recent methodological advances of the MSFD TGML, and on the results of the project's testing phase.

PREPARED BY

THE INTERREG MED PLASTIC BUSTERS MPAs PROJECT

14.1 Species sampling

The presence and impact of marine litter in Mediterranean seabirds (*Calonectris diomedea, Puffinus yelkouan, Ichthyaetus audouinii*) can be investigated and/or monitored in animals dead, on beaches or from accidental mortalities such as long-line victims. Live animals could be sampled in the rescue centres during the hospitalization or in their colonies. In case of doubt about the species identification, refer to identification guide (e.g. www.cites.org) or an expert in the field.

The seabirds above indicated are protected species, therefore only authorized people can handle live and dead animals or parts of them. Upon finding the animal, its management and recovery should be reported and coordinated with the responsible Authorities. Note that a CITES permit is asked if a specimen or sample has to be sent/received.

14.2 Data to be recorded

Taking pictures of the animal before handling it is key to verify the circumstances of the finding and to *a posteriori* confirm or clarify the noted information if doubts or difficulties are encountered in identifying the species, the lesions, the state of the individuals and the elements responsible for the interaction.

All the sea birds' data should be noted down in a sampling sheet (see paragraph 14.11).

14.3 Conservation/health status of the organism

With regards to the health status of the organism, two cases are possible: the seabird may be live, or dead. Overall, 5 different situations can be observed:

- Level 1: the animal is live. In this case biological samples (blood, plasma, faeces) can be removed for biomarkers and chemical analyses. In animals that have just died (< 2 hours post mortem), GI is adequate for litter ingestion analysis and other tissues (muscle, liver) can be used for biomarker and chemical analyses.
- Level 2: Fresh carcass (< 24 hours post mortem), adequate for litter ingestion analysis from necropsies and chemical analysis.
- Level 3: Moderate decomposition, adequate for litter ingestion analysis from necropsies and chemical analysis.
- Level 4: Advanced decomposition, adequate for litter ingestion analysis from necropsies and chemical analysis.
- Level 5: Mummified or skeletal remains. In this level individuals have usually lost their gastro-intestinal material and thus, the analysis of litter ingestion is not possible.

Discovery circumstances

Note the circumstances among the 2 categories:

- Stranding: animal found stranded on the beach or in the shoreline.
- Dead at the recovery centre: the animal arrived live but died during its hospitalization.

Possible cause of morbidity and mortality, type of impact

If possible, the type of interaction with human activities and impact observed or suspected on dead or live stranded individuals should be deduced from external or organs observations during the necropsy and complemented with veterinarian examinations.

Also, an inspection of the oral cavity should be conducted for the presence of foreign material. Then a choice among the following categories should be made and the notes and remarks box should be completed with the help of the pathologist (if this is requested):

- Bycatch/Fisheries related: ingested hook, individual trapped in a fishing gear, individual drowned in a fishing gear.
- Entanglement in litter: entanglement in litter other than related to fishing activity. Please fill the column "Entanglement type" and "Litter causing entanglement".
- Ingestion of litter: digestive obstruction or occlusion, perforation, or other impacts.
- Anthropogenic trauma.
- Natural trauma or natural disease.
- Oils: Ingestion or external impregnation with oils.
- Unidentified: Impossible to know the cause of death/stranding, no remarkable damages, injury or disease.
- Other: Please specify in the column "Notes".

Main injuries

In case of injuries, the main type of injury (fracture, amputation, sectioning, abrasion or other) and the affected body part should be reported.

If the individual has been found entangled in litter, the type of material in which the seabird was found should be specified.

Biometric Measurements

Several basic and body lengths (Culmen Lenght -CL, Wing Length -WL, Head Length -HL, Bill Depth -BD, Weight, Tarsus Length -TL) can be measured (in centimetres, precision 0.01 cm), as well as the weight (in kilograms, precision 0.01g). If possible, the sex (male or female) should be noted, which is determined by gonads observation. Otherwise, specify by NI (for Not Identified). Age, the only variable found to influence litter quantities in stomach contents, is largely determined based on development of sexual organs (size and shape) and presence of *Bursa of Fabricius*.

14.4 Protocol for dead seabirds

Dissection procedure

Immediately after sampling, label the animal (unique ID for each individual) and transport it to the laboratory in ice containers and store at -20 °C until dissection for litter and contaminant analysis. Thaw the animal in the laboratory at room temperature, then dissect out gastrointestinal tract (GI) for litter analysis and the other tissues for contaminant analyses.

Tissue collection

For contaminants assessment, about 10g of each of the following tissues should be collected, wrapped in aluminum foil and stored at -20 °C: muscle, liver, subcutaneous fat from different parts of the body, kidney. Each tissue stored in aluminium foil must be labelled with the standard identification code of the animal (unique ID for each individual).

Gut content analysis

The stomachs of dissected birds are to be opened by scissors or scalpel. Stomach contents should be 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 to be used because smaller meshes become easily clogged with mucus from the stomach wall and with food-remains.

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

14.5 Protocol for live seabirds

The sampling of live seabirds can be applied in seabird colonies (free-ranging animals) or in animals hospitalized in rescue centres.

In seabird colonies, nests can be difficult to access. Safety requirements for boating, climbing and hiking should be followed. In some risky conditions, despite protocols being simple, only experts should be asked to take samples.

Moreover, seabird welfare and safety should be a priority for coordinators and operators, and unnecessary stress to birds should be avoided. Some precautions, such as cover bird head, avoid noise, exclude from sampling nests in unfavourable conditions, and fast sampling procedures should be considered case by case.

Sample collection

- The collection of biological samples on live organisms should be made by authorized personnel.
- Biological tissues (blood, oil gland secretion, faeces, and abandoned eggs) must be collected, processed, and immediately stored in liquid nitrogen or dry ice. Each tissue must be stored in aluminum foil and labelled. All the biological samples collected are to be used for biomarker and chemical analyses.

Blood sampling

- Blood (the amount depends on the size of the animal ranging from about 50 ul to 2 ml) should be collected from a brachial vein using an insulin syringe. The brachial/ulnar vein is located just beneath the ventral surface of the humeral-radialulnar joint. Extend the wing, possibly with the aid of a collaborator, and clear the area of feathers around the ulnar-humoral joint using a cotton ball soaked in distilled water until the brachial vein is visible.
- Then, with a 23-25-gauge needle (internal diameter of about 0.3 mm) kept in orthogonal sense with respect to the vein, gently prick. If the blood flows poorly, insert the needle slowly into the vein and use a needle bore size more appropriate.
- Once the blood is flowing, remove the needle and use the syringe for collecting it (Owen, 2011). To stop the blood from flowing, press on the puncture site using cotton wool for half a minute. Allow the wing to fold naturally against the body, securing in that position to prevent flapping. The blood should be transferred into a solvent-rinsed glass vials (10-5 ml) with Teflon caps containing heparinized saline (heparin sodium) and the tubes gently mixed.

Figure 14-1. *Blood collection from the brachial vein in a specimen of Calonectris diomedea.*

One drop of blood is enough to obtain a **blood smear**. Each sample must be done in double. Once the blood is collected using a syringe, a drop of blood must be transferred to each slide. The blood smear should be performed by a different operator from who makes the blood collection, as blood immediately coagulates and, contemporary, bleeding must be stopped. The blood smear is done using a third clean slide as shown in the picture. Dry the slides at the air. Slide fixing shall be done the same day of sampling, after the slide is completely dry. Immerse the slides in ethanol for 10 minutes and dry the slides in the air, then place the slides in the appropriate slide box.

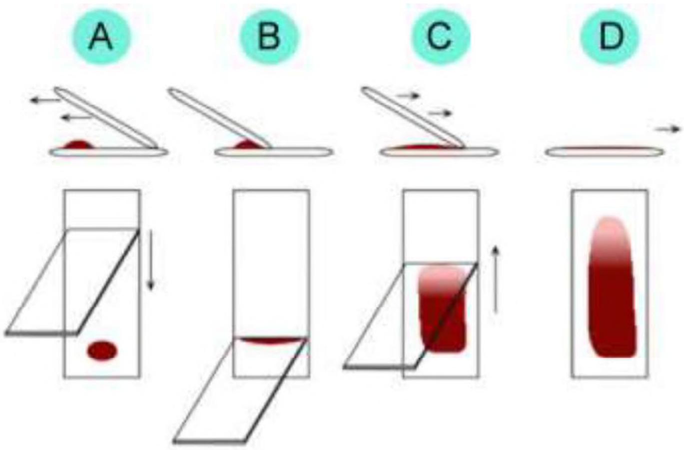


Figure 14-2. *Blood smear collection.*

A part of the blood (2 ml) is transferred into smaller (2 ml) centrifuge tubes and centrifuged at 5000 x g for 5 minutes for the separation of **plasma** and immediately transferred into smaller plastic tubes (0.5 ml) containing a small amount of antiprotease cocktail (5 µl). Placed into ice dry or liquid nitrogen (make a small hole in the upper part of the tubes to avoid “explosion” when taking them out of the liquid nitrogen).

A part of the **whole blood** (1 ml) will be stored without centrifugation in plastic tubes in liquid nitrogen or dry ice or -20 °C.

500 uL of whole blood and 500 uL of mixture (RPMI and DMSO conservation mix, 80:20) will be transferred into smaller (2 ml) centrifuge tubes and placed into liquid nitrogen or dry ice for biomarker analysis (comet assay).

Oil gland secretion sampling

This minimally intrusive protocol aims to collect a small quantity of oil from the uropygial gland of live birds, in order to detect contaminants (phthalates).

- Once the bird is kept in hand, gently massage the preen gland at the upper base of the tail. With bare hands, give a gentle squeeze after massaging the gland so that a small amount of oil can be obtained.
- The gland secretes a waxy substance, rather than a fluid oil as one could expect. Using a pair of metal tweezers that have not been in contact with plastic, remove a clean cotton wool from a glass jar.
- Gently massage the oil gland and wipe cotton wool over the gland 1-2 times to transfer the oil gland exudate to the cotton wool. Do this without touching latex gloves or other plastic items. Then, place the cotton wool back in a glass jar. Seal and label the jar (Hardesty et al., 2015).

Figure 14-3. *The uropygial gland in a seabird (from CSIRO, 2013).*

- After sampling, be careful with the glassware containing samples. To transfer samples from field to the laboratory, it may be useful to protect vials with packaging material, avoiding any plastic products even if vials are sealed. Use leather, corn or paper materials.

Faeces sampling

Faeces can be taken into or next to each nest in the colony. Fresh faeces ejected during bird handling can be collected too. Dry, almost dry or fresh faeces should be collected using a teaspoon or spatula (about 1 g needed). Put them in a piece of aluminum foil which will be closed as an envelope (or in an Eppendorf). Beware that samples faeces are not contaminated with the soil or other external materials and do not mix excrement samples from different nests, or sites. After sampling, freeze samples at -20 °C (within 24-48 hours) to detect possible presence and effect of litter ingestion.

14.6 Litter analysis and classification

Macrolitter detection

- Sort prey or litter items from the bird stomach into separate categories under a stereomicroscope, taking care of recording their weight.
- Measure the size of litter items and classify litter categories.

In addition, the following parameters should be recorded:

- For all categories: the dry mass (grams, precision 0.01 g) of each category; dry the sample at room temperature during 24 h minimum or in a stove at 35°C during 12 h.
- For litter categories only: the number of fragments and items in each category; a fragment is a piece of litter that can be identified while an item is a set of fragments that seem to originate from the same piece of litter
- For the plastic litter categories only: the total number of plastic fragments per colour category, with specifics as follow:
    - Total number of white-transparent plastic fragments;
    - Total number of dark coloured plastic fragments (black, blue, dark green…);
    - Total number of light-coloured plastic fragments (cream, yellow, pink, light green…).
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

Microlitter detection

- Examine the content >1mm in the Petri dish under a stereomicroscope for particles resembling microplastics. Cover the Petri dish with glass lids during observation not to contaminate the sample.
- Photograph, count and record the type, colour and maximum length of microplastic particles using image analysis software.
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

The limit detection for MSFD is 1 mm. Building on the findings of the testing phase of the Plastic Busters MPAs project, it's recommended to also examine the fraction 0.1-1 mm.

Litter categories

Categorize marine litter according to the categories showed in Table 14-1. The categorization 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 in most cases, particles cannot be unambiguously linked to particular objects. But where is possible, under notes in datasheets, the items should be described and assigned a litter category number using the "Joint List" developed by the TSG ML group (Fleet et al., 2021). In addition, it is important to measure and quantify also natural items (food and/or no food).

Table 14-1. *Categories for classification of items for sea birds (MSFD Protocol for the monitoring of litter ingested by seabirds, 2022).*

BIOTA categories for contents of digestive tract			
PLA	**PLASTIC**	**acronym**	all plastic or synthetic items: note number of particles and dry mass for each category
IND	**pellets**	**ind**	industrial plastic granules (usually cylindrical but also oval spherical or cubical shapes exist)
	probab ind?	**pind**	suspected industrial, used for tiny spheres (glassy, milky, ...) (= microbeads)
USE	**sheet**	**she**	remains of sheet, eg from bags, cling-foil, agricultural sheets, rubbish bags etc
	thread	**thr**	threadlike materials, eg pieces of nylon wire, net-fragments, woven clothing; includes 'balls' of compacted material
	foam	**foam**	all foamed plastics, polystyrene foam, foamed soft rubber (as in matrass filling), PUR used in construction etc
	fragments	**frag**	fragments, broken pieces of thicker type plastics, can be bit flexible, but not like sheetlike materials
	other	**Poth**	any other, incl elastics, dense rubber, cigarette-filters, balloon-pieces, softairgun bullets, objects etc. DESCRIBE!!
RUB	**OTHER RUBBISH**	**acronym**	any other nonsynthetic consumer wastes: note number of particles and (in principle) dry mass for each category
RUB	**paper**	**pap**	newspaper, packaging, cardboard, includes multilayered material (eg Tetrapack pieces) and aluminium foil
	kitchenfood	**kit**	human food remains (galley wastes) like onion, beans, chickenbones, bacon, seeds of tomatoes, grapes, peppers, melon etc
	other rubbish	**rubvar**	other various rubbish, like processed wood, pieces of metal, metal air-gun bullets; leadshot, paintchips. DESCRIBE
	FISHHOOK	**hook**	fishing hook remains (NOT FOR HOOKS ON WHICH LONGLINE VICTIMS WERE CAUGHT - THOSE UNDER NOTES)
POL	**POLLUTANTS (INDUS/CHEM WASTE)**	**acronym**	other non-synthetic industrial or shipping wastes (number of items and mass per category (wet 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=0.0001g if other particles smeared with tar but cannot be sampled separately)
	paraf/chem	**chem**	lumps or soft mush of unclear paraffin, wax like substances (NOT stomach oil!); if needed estimate mass by subsampling
	featherlump	**confea**	lump of feathers from excessive preening of fouled feathers (n=1 with drymass) (NOT for few normal own feathers)
FOO	**NATURAL FOOD**	**foo**	various categories, depends on the species studied, and aims of study
NFO	**NATURAL NON FOOD**	**nfo**	anything natural, but which cannot be considered as normal nutritious FOOD for the individual

Collection of data

For each organism, an assessment is made of:

1. Frequency of occurrence (%) of ingested macro and microlitter for each species, calculated as the percentage of the individuals examined with ingested macro- and microplastics.
2. Abundance (N) of macro and microlitter ingested per individual (average number of items/individual) for each species, calculated as a total and per category. Since currently there are inconsistencies in the literature in reporting abundance of ingested litter, it is recommended to report average number of items per individual considering both all individuals examined and only individuals found with ingested macro and litter.
3. Total dry weight (g) of the detected waste expressed on grams (precision: second decimal place). This weight refers to each single category found in a specific organ (or faeces) of the specimen.

   Other information as colour of items, polymer of the different items (at least 10% of the total items) and different incidence of litter in oesophagus, stomach and intentine, incidence and abundance are useful for research and impact analysis.

14.7 Analysis of plastic tracers and PBTs

Plastic additives

The compounds to be detected in different tissues/fluid are:

- *Phthalates:* a group of chemicals widely used as additives to make plastics more flexible and harder to break; they can interfere with endocrine system (Baini et al., 2017).
- *Bisphenol A*: used in the production of polycarbonate, can have endocrine disrupting effects (Crain et al., 2007; Halden, 2010; Oehlmann et al., 2009) and the styrene and polyvinyl chloride monomer, used in the production of polystyrene and polyvinyl chloride (PVC), can be carcinogenic and/or mutagenic (Lithner et al., 2011; Papaleo et al., 2011; Xu et al., 2004).
- *Polybrominated diphenyl ethers:* they belong to the group of brominated flame retardants (BFRs), which are used in various polymeric materials such as plastic parts, resins, textiles, and other substrates to reduce their fire hazards (BSEF 2003; Król et al. 2012).

Persistent, bioaccumulative and toxic substances (PBTs)

In addition to the plastic additives that may leach from plastics when released into the marine environment, plastics tend also to adsorb in their surface persistent bioaccumulative and toxic substances (PBTs) (e.g. organochlorine compounds OCs, PAHs and PBDEs) and metals (e.g., lead, copper and cadmium) that are present in the seawater.

Depending on the compounds and the tissue to be analysed, different methods should be applied to detect the presence of plastic-related contaminants in the fish species (Annex V).

14.8 Biomarkers analysis

The toxicological effects associated with the presence of marine litter can be evaluated using a set of diagnostic and prognostic methodologies, by means of biomarkers. A non-exhaustive list of existing biomarker approaches and plastic tracers' contaminants that are usually applied in seabirds analyses is reported in Annex V.

Biomarkers have been selected on the basis of the level of biological responses and in relation to the main effects related to marine litter/microplastics ingestion. The selected biomarkers can diagnose different impacts related to: a) physical damages/effects of marine litter, b) exposure to/effect of chemical tracers, and c) exposure to/effect of adsorbed chemicals.

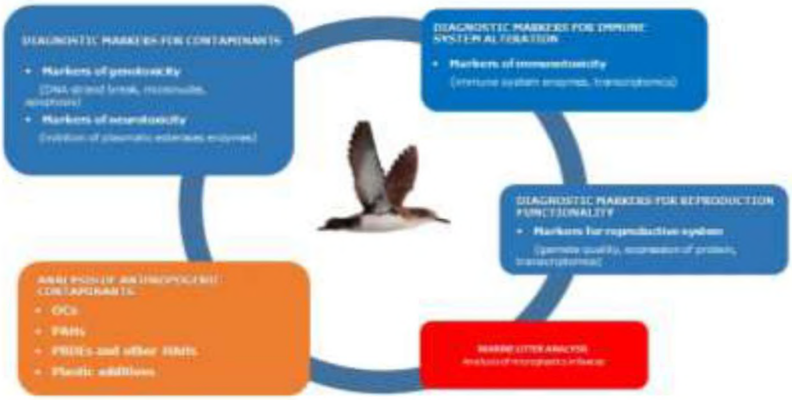
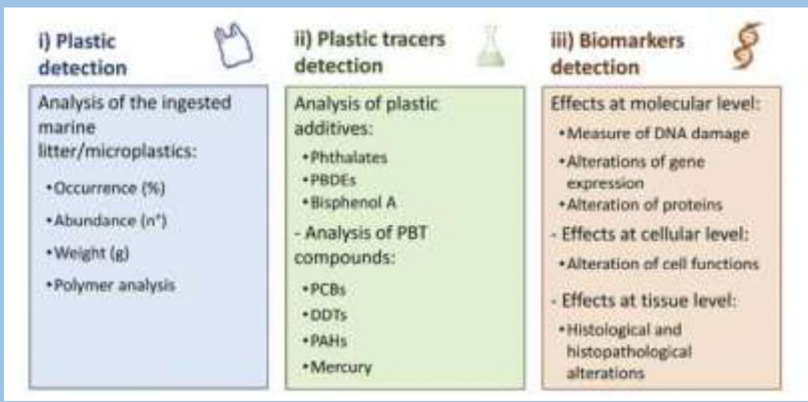


Figure 14-4. *A three-fold approach to detect the marine litter presence and impacts to seabirds species.*

Starting from this initial list and building on the findings of the testing phase of the Plastic Busters MPAs project, the most suitable diagnostic tools to detect the presence and impact of ML on seabirds are proposed here below.

Table 14-2. *Main diagnostic tools selected in the PLASTIC BUSTERS MPAs project to detect the presence and impact of ML in seabirds.*

SEABIRDS: MAIN DIAGNOSTIC TOOLS SELECTED IN THE PLASTIC BUSTERS MPAs PROJECT



1. Analysis of gastrointestinal content: litter analysis and classification (stranded organisms)
2. Analysis of Faeces: Litter and microlitter analysis and classification
3. Analysis of Nest: Litter and microlitter analysis and classification
4. Analysis of plastic tracers: phthalates
5. Analysis of biological end-points: ENA assay, porphyrins, vitellogenin, complement system, LPO

14.9 Materials & Equipment for sampling

The following material and equipment are necessary for the correct application of the protocol:

- Boots
- Camera
- Clamps (at least 6) or roast wire
- Clips with claws
- Containers for samples (Bottle/zipped bags)
- Cooler
- Cut-resistant gloves
- Garbage bag
- Glasses and protective mask or shield
- Nitrile Gloves
- Integral protective suit
- Measuring decimetre
- Measuring tape
- Metal containers
- Metal spoon
- Pen
- Permanent marker
- Sampling sheets
- Scalpel
- Scissors
- Sieve with 1 mm mesh
- Sieve with 5 mm mesh
- Transport bins or containers
- Aluminium foil
- Cryoboxes
- Cryovials
- Eppendorf (0.5 ml. 1.5 ml. 2.0 ml)
- Falcon tubes
- GPS
- Liquid nitrogen Dewar (in alternate dry ice)
- Paper and block-notes
- Paper towels
- Pasteurs
- Pencils
- Plastic Sealable bags
- RNAlater
- Ruler
- Scalpels
- Spare batteries
- Thermic bags
- Tweezers

References

Guidance on Monitoring of Marine Litter in European Seas, 2022. A guidance document within the Common Implementation Strategy for the marine Strategy Framework Directive - Marine Litter Impact on Biota. MSFD Technical Group on Marine Litter - draft.

Fossi, M.C, Vlachogianni, T., Anastasopoulou, A., Alomar, C., Baini, M., Caliani, I., Campani, T., Casini, S., Consoli, P., Cillari T., D'Alessandro, M., Deudero, S., Galgani, Galli M., F., Kaberi H., Panti, C., Pedà, C., E. Romeo, T., Scotti, G., Tsangaris, C., Zeri, C., 2019. Toolkit for monitoring marine litter and its impacts on biodiversity in Mediterranean MPAs. Interreg Med Plastic Busters MPAs project (D.3.3.2).

Monitoring Marine Litter in stranded/dead seabirds

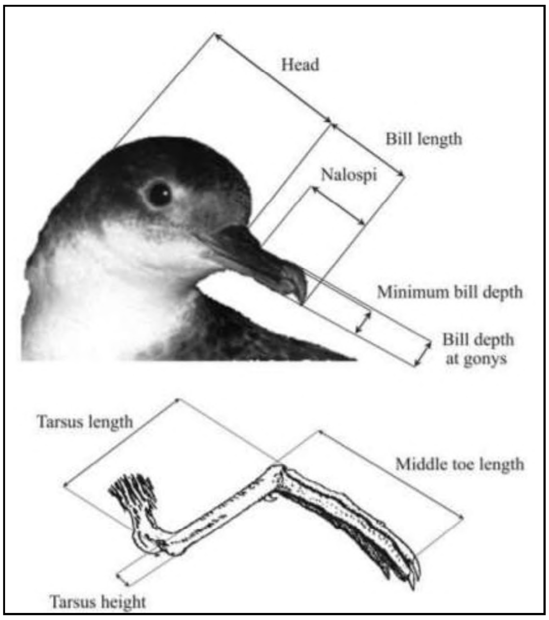
Species:		**ID code:**	
Ringing code:			
Location/Country:		**Latitude**	**Longitude**
Discovery circumstances	☐ Stranding	☐ Dead at recovery center	
Cause of mortality (*Please specify according to the toolkit*)			
Date of discovery			
Date of necroscopy			

Animal body condition					
Conservation status	☐ Level 1	☐ Level 2	☐ Level 3	☐ Level 4	
Main injuries	☐ No injuries ☐ Abrasion	☐ Fracture	☐ Amputation	☐ Sectioning	
Affected parts	☐ Wings	☐Tail	☐Neck	☐ Head	☐ Other

Collected tissues		**N°. of aliquots**
Muscle		
GI tract		
Liver		
Kidney		
Sub-cutaneous fat		

Necroscopy performed by:

Name and Institution:



Measurement			
Sex	☐ Male ☐ Female ☐ Not Identified		
Culmen **L**ength (CL)		**B**ill **D**epth (BD)	
Wing **L**ength (WL)		Weight (kg)	
Head **Length** (HL)			

Note and remarks:

Monitoring Marine Litter in live seabirds

<table>
<tr><td colspan="2">Species:</td><td colspan="2">ID code:</td></tr>
<tr><td colspan="4">Ringing code:</td></tr>
<tr><td colspan="2" rowspan="2">Location/Country:</td><td>Latitude</td><td>Longitude</td></tr>
<tr><td></td><td></td></tr>
<tr><td>Discovery circumstances</td><td colspan="3">☐ Stranding ☐ Dead at recovery center</td></tr>
<tr><td>Cause of morbidity
(Please specify according to the toolkit)</td><td colspan="3"></td></tr>
<tr><td>Date of discovery</td><td colspan="3"></td></tr>
</table>

<table>
<tr><th colspan="4">Measurement</th></tr>
<tr><td>Sex</td><td colspan="3">☐ Male ☐ Female ☐ Not Identified</td></tr>
<tr><td>Culmen Length (CL)</td><td></td><td>Bill Depth (BD)</td><td></td></tr>
<tr><td>Wing Length (WL)</td><td></td><td>Weight (kg)</td><td></td></tr>
<tr><td>Head Length (HL)</td><td></td><td></td><td></td></tr>
</table>

<table>
<tr><th colspan="4">Collected tissues</th></tr>
<tr><td>Whole blood</td><td></td><td>Feathers</td><td></td></tr>
<tr><td>Plasma</td><td></td><td>Uropygial gland</td><td></td></tr>
<tr><td>Liver</td><td></td><td>Excreta</td><td></td></tr>
<tr><td>DMSO:RPMI conservation mix</td><td></td><td>Egg</td><td></td></tr>
</table>

ANNEX V

Table A-7. *Tissues and methods to be used to detect plastic-related contaminants in seabirds.*

	CHEMICAL COMPOUND	TISSUE/SAMPLE	ANALYTICAL METHOD
PLASTIC ADDITIVES	**Phthalates**	Fat, muscle, liver	Baini et al., (2017), Fossi et al., (2016), Savoca et al., (2018)
		Blood	Takatori et al., (2004)
		Oil gland secretion	Hardesty et al., (2015), Provencher et al., (2020)
	Bisphenol A	Muscle	Ballesteros-Gómez et al., (2009)
		Fat	Xue et al., (2016)
		Blood	Cobellis et al., (2009)
	Polybrominated diphenyl ethers	Fat, muscle, liver, egg, blood	Muñoz-Arnanz et al., (2016), Sühring et al., (2022)
ADSORBED CONTAMINANTS	**Polycyclic aromatic hydrocarbons**	Fat, muscle, liver, blood	Marsili et al., (2001)
	Organochlorine contaminants	Fat, muscle, liver, blood	Marsili and Focardi, (1997), Sühring et al., (2022)
	Mercury	Blood, kidney	Correa et al., (2013), (Espín et al., 2012)

Table A-8. *Effects measured in seabirds by the biomarker approach.*

EFFECT	TISSUE	TEST
GENOTOXICITY	Blood	Comet assay (Molino et al., 2019) (*) Mn test (Bolognesi et al., 2006) ENA assay (Casini et al., 2018); (Pacheco and Santos, 1997)
OXIDATIVE STRESS	Liver, kidney	CAT, GST, LPO, GPX, GR, GSH (Yu et al., 2018) (*)
	Plasma	LPO (Fossi et al., 2016), Casini et al., 2018) CAT (Fossi et al., 2013)
IMMUNOTOXICITY	Blood	Total and differential white blood cells (WBC) count (Casal and Orós, 2007; Davis et al., 2008; Caliani et al., 2019) H:L ratio (Caliani et al., 2019) Respiratory burst (Secombes, 1990; Caliani et al., 2019) TAS assay (Miller et al., 1993; Caliani et al., 2019) Lisozyme enzyme (Keller et al., 2006; Caliani et al., 2019) casp8, casp9, TRAF (Karami et al. 2017; Mathieu-Denoncourt et al., 2015) (*)
REPRODUCTION	Plasma, Gonads	CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015) (*) Vitellogenin (Fossi et al., 2004)
	Plasma	Vitellogenin (Herbst et al., 2003) CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*)
HISTOPATHOLOGY INFLAMMATION AND MORPHOLOGY	Liver, kidney	Histopathology,histology (Pedà et al. 2016; Karami et al. 2017; Batel et al., 2018) (*)
XENOBIOTIC METABOLISM AND BIOTRANSFORMATION	Blood, excreta, liver	CYP1A; AHR, CYP3A (Fossi et al. 2014, Panti et al. 2011; Rochman et al., 2013) (*) Porphyrins (Guerranti et al., 2014) (*)
NEUROTOXICITY	Brain, muscle, plasma	AChE, BChE (Barboza et al., 2018) (*)

CELLULAR STRESS	Blood, liver, kidney	PPARA, PPARG, HSP70, GPX, E2F1 (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*) Gamma glutamyl transferase (GGT) (Nematdoost Haghi and Banaee, 2017) (*) Cortisol and corticosterone (Flower et al., 2015) LDH (Nematdoost Haghi and Banaee, 2017) (*)

(*) effects detected after laboratory or field exposure with MPs or plastic tracers.

15. Methodology for monitoring presence and effects of marine litter in marine mammals

This document describes the methodological approach for monitoring the presence and effects of marine litter in marine mammals, which has been developed within the framework of the Interreg Med Plastic Busters MPAs project, building on the most recent methodological advances of the MSFD TGML, Barcelona Convention CORMON, ACCOBAMS/ASCOBAMS, and on the results of the project's testing phase.

PREPARED BY

THE INTERREG MED PLASTIC BUSTERS MPAs PROJECT

Interreg Mediterranean

PLASTIC BUSTERS MPAs

15.1 Sampling strategies and analyses

The methodology of this tool follows the methods described in the literature based on the work of responsible bodies such as the IWC and ACCOBAMS/ASCOBANS for monitoring of micro and macro litter ingested by marine mammals and it has been adapted within the framework of the Interreg Med Plastic Busters MPAs project to address the recent advances in the field.

The ingestion of macrolitter and microlitter by Mediterranean marine mammals such as deep diver cetaceans species (*Physeter macrocephalus, Ziphius cavirostris),* coastal and pelagic odontocetes (*Tursiops truncatus, Stenella coeruleaolba, Delphinus delphis, Grampus griseus, Globicephala melas*), mysticete (*Balaenoptera physalus*) and pinniped species (*Monachus monachus)*, and the potential related effects can be investigated and/or monitored in:

- **Dead organisms** which may have been stranded ashore, found at sea, etc.
- **Free ranging organisms** that have been sampled at sea.

The marine mammals are protected species, therefore only authorized people can handle live and dead animals or parts of them. Upon finding the animal, its management and recovery should be reported and coordinated with the responsible Authorities. Permits released by national competent authorities are required for cetacean biopsy sampling. Note that a CITES permit is asked if a specimen or sample has to be sent/received.

15.2 Protocol for dead animals

Protocols for the analysis of marine litter in stranded marine mammals were developed according to the existing protocols for other marine taxa (Lusher et al., 2017, 2018, Fossi et al 2018). The methodology proposed in this document has already been integrated into the related protocol which was developed by a Joint ACCOBAMS and ASCOBANS document "*Best practice on cetacean post mortem investigation and tissue sampling "* (Ijsseldijk et al., 2019).

Figure 15-1. *A stranded cetacean.*

Conservation status or decomposition level

When approaching to a dead organism, the conservation status is important for the diagnosis of the cause of death and for the analysis to be carried out on it.

With regards to the status of the dead cetaceans, 5 different situations can be observed:

Figure 15-2. *Condition status and codes for stranded organisms.*

- **Level 1**: the animal is found live or deceased at most by 2 h, adequate for *litter ingestion investigation, chemical analysis and biomarkers analyses.*
- **Level 2** (Death within 24 h); normal appearance with minimal damage from scavenger animals; normal smell; minimal skin dehydration and rippling of the skin, and apparent mucous membranes; clean and shiny eyes; uninflated carcass, tongue and penis not protruding. *Adequate for litter ingestion and chemical analyses.*
- **Level 3**: Whole carcass, with evident swelling (tongue and penis protruding); skin not integrated with detachment areas; possible damage from scavenger animals; slight characteristic smell; apparent dry mucous membranes; eyes introflexed or missing. *Adequate for litter ingestion and chemical analyses.*
- **Level 4**: The carcass may be intact, but collapsed; wide areas of skin disepithelialization; severe damage from opportunistic animals; strong smell; muscles and blubbers easily removable and detachable from the bone; liquefaction of internal organs; allows to measure biometric data and assess the *presence/absence of ingested plastic and chemical analyses*.
- **Level 5**: Often with dehydrated skin and dry over the bones; completely dry; the analyses of litter ingestion or chemicals are not possible.

Below is reported the Table 15.1 where the conservation level of the specimens is related to the different types of applicable investigations, including contaminant investigations and identification of the Marine Litter ingested.

Table 15-1. *Recommendation for tissue sampling considering carcass DCC. Shading: green* ***V*** *indicates the process is of potential use in carcasses of the indicated DCC; grey (****V****)indicates that there may be limitations and red* ***V*** *indicates the procedure is not recommended/very unreliable, due to post mortem autolysis (From Best practice on cetacean post mortem investigation and tissue sampling" (IJsseldijk et al., 2019).*

Analytical procedure	DCC 1	DCC 2	DCC 3	DCC 4	DCC 5	Comments/recommendations
Genetics	✓	✓	✓	✓	✓	For DCC4 or 5: paleopathological procedures may be required on account of degraded DNA (eg extracting DNA from bone medulla)
Diet and marine debris	✓	✓	✓	✓	(✓)	If GIT is not intact, eg from post mortem scavenger damage, results are compromised
Age determination	✓	✓	✓	✓	(✓)	
Fatty acids and stable isotopes	✓	✓	✓	✓	(✓)	Depending on analysis planned
Parasitology	✓	✓	✓	✓	(✓)	Depending on analysis planned
Morphometrics	✓	✓	✓	(✓)	(✓)	Girth measurements can be disrupted by bloating due to autolysis in DCC4-5
Gross pathology	✓	✓	✓	(✓)	(✓)	Recommended for DCC4-5 in cases of forensic investigation
Reproductive studies	✓	✓	✓	(✓)	✗	
Toxicology	✓	✓	✓	(✓)	✗	Depending on pollutants. DCC1-2 for biomarker investigation.
Ear investigation	✓	✓	✓	✗	✗	Inner ear analysis specifically: DCC1, histopathology of fixed ears possible up to DCC3
Microbiology	✓	✓	(✓)	(✓)	✗	Depending on analysis planned. For DCC3-4 microbiology can still be worthwhile for detection of certain bacteria and fungi using specific culture methods. Should a septicaemia be suspected in DCC3-4 animals, then microbiological investigations should be undertaken on the kidney, as this is resilient to microbial post mortem invasion using specific culture methods.
Histopathology	✓	✓	(✓)	(✓)	✗	Recommended for DCC4-5 in cases of forensic investigation
Virology	✓	✓	(✓)	✗	✗	Depending on analyses planned.

Biotoxins	✓	✓	(✓)	✗	✗	
Gas bubble analysis	✓	✓	✗	✗	✗	If this procedure is conducted: it should be done first, before undertaking further assessments and dissections, particularly prior opening any part of the vascular system or removing the head.
Serology	✓	(✓)	(✓)	✗	✗	Advisable both on blood serum and on cerebro-spinal fluid, the latter of which should be collected as soon as possible. In heavily autolyzed specimens, alternatives are "juice" obtained from skeletal muscle or lung, vitreous humour or pericardial fluid
Clinical chemistry	✓	✗	✗	✗	✗	Vitreous humour is a possible option in decomposed cases . Care is needed however to ensure sufficient baseline data are available for the analyte in the species under investigation.

Discovery circumstances – including entanglement and bycatch

Note the **circumstances** among the 5 categories:

- *Stranding**: Animal found stranded on the beach or in the shoreline,
- *By-catch*/Fisheries*: Animal accidentally captured by fishers (e.g. ingestion of a hook, trapped in a net, brought back by fishers, etc.) during fishing operations.
- *Found at sea*: Animal discovered on sea surface.

** If possible, the <u>type of interaction with human activities and impact observed or suspected on dead or live stranded individuals</u> should be deduced from external or organs observations during the necropsy and complemented with veterinarian examinations. Also, an inspection of the oral cavity should be conducted for the presence of foreign material.*

Biometric Measurements and sex determination

Several basic and optional body lengths can be measured (in centimetres, precision 0.01 cm), as well as the weight.

The sex (male or female) should be noted, which can be determined by observation of sexual characters. Otherwise, specify by NI (for Not Identified).

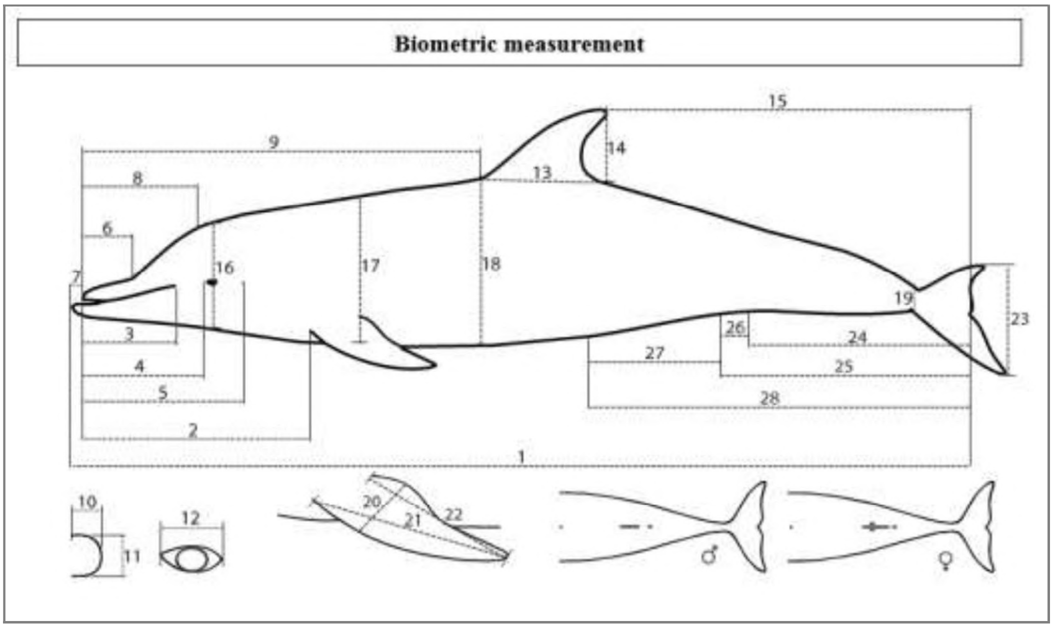


Figure 15-3. *Cetacean biometric measurement (from http://mammiferimarini.unipv.it/).*

Necroscopy and Health status

Necroscopy should be performed under the authorization needed at National level and with the presence of a veterinarian.

The body condition of a cetacean can be assessed by looking along the dorsal axis of the animal (poor, fair, good). The dorsal muscle mass (epaxial muscle) to either side of the dorsal fin of a robust animal will be rounded or convex. A thin animal will have a slight loss in epaxial muscle girth and could have a minor sunken aspect to the dorsal-lateral body. An emaciated animal will have a greater loss of epaxial muscle girth and will be concave down the dorsal-lateral body. Emaciated animal may also have more prominent indentation at the nape.

In addition visual inspection of the animal's fat reserves at the dorsal fin is recommended. Choose among the 3 categories:

- Thin;
- Fat;
- Normal.
- Not recorded (NR)

Extraction of the gastrointestinal system:

- Expose the gastrointestinal system (GI) by removing all excess attached tissues, the heart and liver of the animal. Clamp the oesophagus proximal to the mouth and clamp the colon, the closest to the anal orifice.
- Remove the entire GI and place it on the examination surface or isolate the different portions of GI (oesophagus, stomach, intestines) by strangling and cutting between 2 clamps the gastro-oesophageal sphincter and the pyloric sphincter. This operation is easier if done by at least 2 operators.
- During the whole procedure, airborne contamination should be prevented as much as possible.

Gut content analysis and marine litter isolation

- Before opening up the digestive tube, examine the outer wall to observe possible perforations by foreign bodies or areas of necrosis. Also, note any eventual secondary lesions, particularly a peritonitis following on a perforation of the digestive tube, an invagination of the digestive tube, an occlusion, etc. Photograph every lesion observed, taking care to get an overall view as well as close-up (macro-lens) photographs. Pictures must be stored referring to the code corresponding to the animal examined, describing the lesion in the description of the subject.
- The three parts of the gastrointestinal system (i.e. oesophagus, stomach, intestines) should be removed by adding a second strangling at the cut edge to prevent spillage of the contents. Each GI section should be opened lengthways using a scissor and slide the material directly out of the section onto a 1mm mesh sieve. The content should be cleaned with abundant tap water to remove the liquid portion, the mucus and the digested unidentifiable matter. Content should be inspected for the presence of any tar, oil, or particularly fragile material, and should be subsequently removed and treated separately. It should be then reported in the column "Notes" of the sampling sheet. All the material should be rinsed, collected in the 1mm sieve, and should be placed in tubes or in zipped bags, reporting the sample code (individual code, respective GI section) and stored at -20 °C, pending the laboratory analyses.
- NOTE: At this stage, for the optional differentiation of litter and microlitter, the material should be slid out of the section directly onto a 5mm mesh sieve superposed on a 1mm mesh sieve. Then, proceed with the rinsing and the storing of the material collected as described above, for both 1- and 5-mm sieves, reporting the samples code (individual code, respective GI section and size class (>5mm or 1-5mm)).
- If possible, follow the protocol developed in Corazzola et al (2021), which allows the simultaneous multidisciplinary analysis of GI by the implementation and standardization of a new methodological approach to the GIT of marine mammals. This protocol allows the collection of samples for different disciplines at the same time, performing the respective analyses, interpret and compare their results in a multidisciplinary way. The compatibility of multiple analyses allows the gaining of more information about the cause of death of stranded marine mammals and to enhance the knowledge of their biology and ecology.

The limit detection for MSFD is 1 mm. Building on the findings of the testing phase of the Plastic Busters MPAs project, it's recommended to also examine the fraction 0.1-1 mm.

Figure 15-4. *A new prototype to isolate macro and microplastics in the gastrointestinal tract of stranded cetaceans (Corazzola et al., 2021).*

Tissue collection

Before sampling the contents of the GI for the subsequent contaminant analysis, collect about 10g of each of the following tissues (level 1-4), wrap them in aluminium paper and store at -20 ° C:

- Muscle
- Liver
- Blubber (include skin) fat taken at the base of dorsal fin
- Kidney
- Brain (if possible include cerebrum and cerebellum)

In case of **Level 1 specimen** (max 1-2h after death):

- Blubber (include skin) for analysis of biomarkers analysis and contaminant analysis: take 10-20g from preserved in aluminum paper,store in liquid nitrogen or dry ice, and then place at -80 °C.
- Liver for biomarkers analysis and contaminant analysis: 10g in aluminum paper, store in liquid nitrogen or dry ice, and then place at -80 °C.Blood for contaminant analysis: 5-10 ml in tubes and store store in liquid nitrogen or dry ice, and then place at -80 °C.Each tissue stored in aluminium foil or Eppendorf must be labelled with the standard identification code of the animal.

15.3 Protocol for free-ranging marine mammals

Figure 15-5. *Skin biopsies: a nonlethal tool for monitoring cetaceans.*

Cetaceans: Remote dart biopsy sampling procedure

A number of successful studies show that cetacean skin biopsies are a powerful nonlethal tool for assessing ecotoxicologic risk in marine mammals and aspects of feeding ecology and food preferences.

Biopsy samples can be taken between the dorsal fin and the upper part of the caudal peduncle upon approaching the animal at a suitable distance and speed as specifically permitted for the species and research project. The skin biopsy needs to be stored immediately in the proper conditions required for intended analyses. Common storage conditions include frozen, as is, in liquid nitrogen, dry ice, and at −80°C and −20° C freezers for long-term storage or stored either cold or at room temperature in cell medium, buffer, or specific reagents (e.g. RNA later). Skin biopsy is a powerful tool for ecotoxicologic studies for the following reasons: (1) it allows collection of a large number of samples across a wide geographic range; (2) it allows collection of sequential samples from the same animal if identified by photo identification or genetics; (3) it is suitable for residue analysis of many contaminants s; (4) it is suitable for several biomarker analyses and cell and organotypic cultures.

Sampling procedure

- Skin biopsies (epidermis and dermis/blubber) from free-ranging dolphins (such as *Tursiops truncatus, Stenella coeruleoalba*) can be obtained using an aluminium pole armed with biopsy tips (e.g. 0.7 cm ø, 3.0 cm length) or with a crossbow and darts.
- Skin biopsies from large odontocete (*Physeter macrocephalus*) or mysticete species (such as *Balaenoptera physalus* or other baleen whales) can be obtained with a crossbow and darts armed with tips (e.g. 0.9 cm ø, 4.0 cm length).

Figure 15-5. *Skin biopsy collection close to the dorsal fin of a fin whale and a striped dolphin.*

This type of sampling requires special permits issued by the competent authorities at national level.

Skin biopsy collection

Once the biopsy has been collected from the animal, it should be processed as soon as possible.

1. Unscrew the tip from the arrow using gloves, put the biopsy in a small bag and write on the bag the code of the animal (put it in the fridge or keep as cold as possible). If there is more than one animal to collect at the same time and you cannot process the biopsy immediately, use a refrigerated bag until the processing.

Figure 15-6. *Arrow and tip (left) and tip on the aluminum pole (right) with the collected biopsy.*

2. Remove the biopsy from the tip using tweezers, paying attention to keep the biopsy entire and put the biopsy on a clean petri dish.

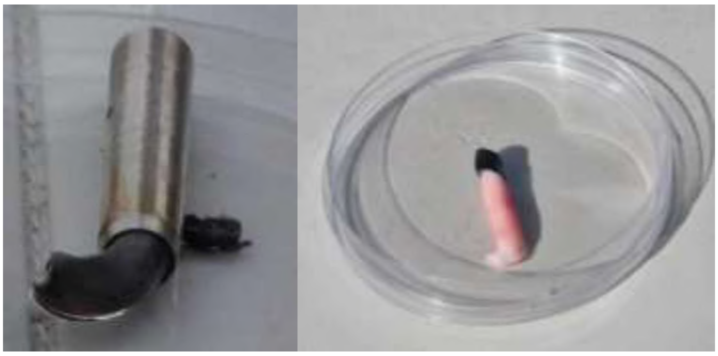


Figure 15-7. *Biopsy sampled: left) biopsy tip, right) biopsy on a Petri dish.*

3. With a clean scalpel cut at least two pieces of skin (about 0.2x0.2 cm each) from the top of the biopsy (yellow squares) and put the separated aliquots of skin in two 0.5 ml Eppendorf. Whenever possible, for larger biopsy, divide the sampled biopsy in 4-5 different aliquots. Wrap up the skin+blubber (red square) in a small aluminium foil and put the biopsy in a 2 ml Eppendorf (contaminants and protein analysis). Organotypic slice cultures should be potentially performed in specialized laboratory.
   Write with a marker the code of the animal on the Eppendorf tubes, and, if possible, put inside the 2 ml tube a small piece of paper with the code of the biopsy written with the

pencil, in order to be sure to not lose the name of the sample. During the operation, fill in the sampling sheet.

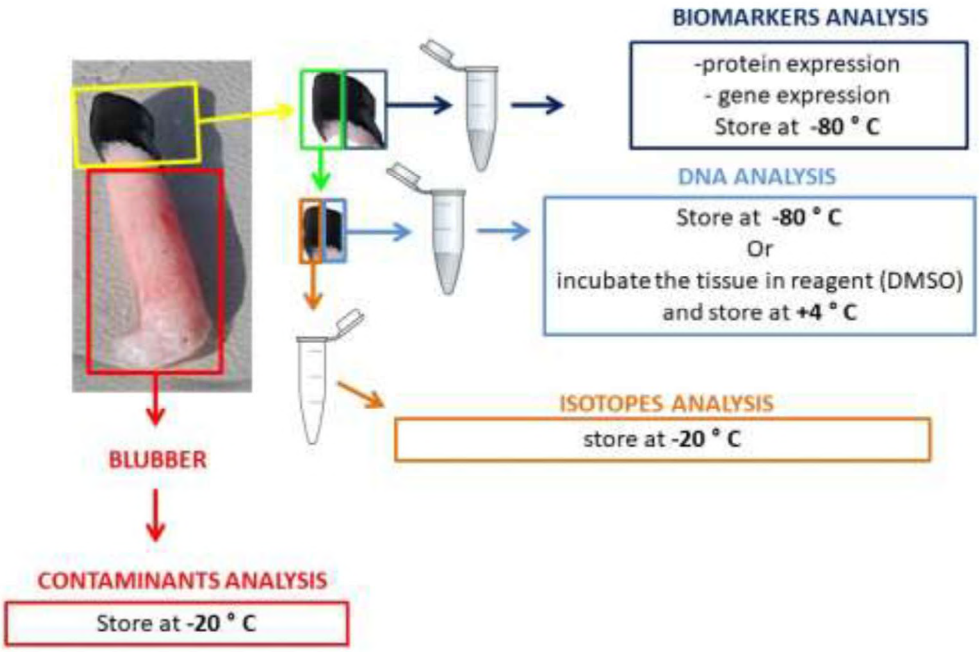


Figure 15-8. *Biopsy with blubber (red square) and dermal part (yellow squares). Operational procedures.*

4. Place the tubes in liquid nitrogen. The samples stored in RNAlater can be kept at room temperature for 24 hours and then stored at +4°C or -20°C for long-term storage.
5. Clean accurately the tips and boil them in freshwater for ten minutes to avoid cross contamination and pathogen transmission among individuals. If boiling the tips is not possible, rinse them with ethanol. Rinse with ethanol also the scalpel and the tweezers.

Storage conditions

For skin biopsy:

- ***Dermal tissues (skin):***
  - 40-60 mg in cryo-vial frozen at -80 °C (protein expression analysis/-omics analysis)
  - 30-50 mg in RNAlater at -20 °C or in cryo-vial frozen directly at -80 °C (gene expression analysis/transcriptomics)
  - 20-30 mg in 20% saturated DMSO with NaCl or in cryo-vial frozen directly at -80 °C (sex determination and genetic analysis)
  - 20-30 mg in cryo-vial frozen directly at -80 °C (stable isotopes analysis)
- ***Blubber tissues (fat):***
  - entire blubber in aluminium foil directly at -80 °C (contaminants analysis)

Faeces collection

- For **free-ranging cetaceans**, faeces collection can be occasional and discontinuous, and generally, available only for fin whale. In case of localization of faeces, they should be collected as much as possible with a net (mesh size 200 µm or less) and put in falcon tubes for subsequent analysis: liquid nitrogen for contaminants and biomarker analysis, -20 °C for litter analysis.
- For **monk seals**, faeces should be entirely collected following the protocol by Lusher and Hernandez-Milian (2018) and stored at -20 °C or dry ice/liquid nitrogen for subsequent litter analysis, contaminant analysis and biomarker analysis. Food remains should be stored in 70% alcohol for diet analysis.

15.4 The threefold approach in marine mammals

After the sampling phases described above (both in stranded and free-ranging animals), the analytical phases can be proceeded, following the methodologies applied in the testing phase of the Plastic Busters MPAs project.

The application of the **threefold approach** can elucidate not only the rate of ingestion in cetaceans, but also the multiple sub-lethal stresses that marine litter ingestion can cause in the short and long term. Each of the three investigation tools that make up the threefold approach can be applied independently or simultaneously using different methods according to the species and whether the animal is stranded or free-ranging.

The threefold approach comprises the following elements:

- **Analysis of gastrointestinal content**: For stranded cetaceans, it is possible to detect the occurrence and rate of marine litter ingestion and any associated pathology through analysis of the gastrointestinal content, with a particular focus on plastics and microplastics.
- **Analysis of the levels of plastic additives, as a proxy for ingestion**: An indirect approach can be used for free-ranging as well as stranded animals. The levels of plastic additives and associated PBT compounds can be measured to evaluate the exposure to marine plastic pollution.
- **Analysis of biological end-points**: Biomarker responses and omics analysis can be used to detect the potential toxicologic effect related to PBT and plastic additives related toplastic ingestion in free-ranging individuals or in stranded organisms up to a few hours after death.

15.5 Litter analysis and classification

Macrolitter detection in stranded organisms

- Sort prey or litter items from the gastrointestinal tract into separate categories under a stereomicroscope, taking care of recording their weight.
- Measure the size of litter items and classify litter.

In addition, the following parameters should be recorded:

- For all categories (litter and other elements): the dry mass (grams, precision 0.01 g) of each category; dry the sample at room temperature during 24h minimum or in a stove at 35ºC during 12h.
- For litter categories only: the number of fragments and items in each category: a fragment is a piece of litter that can be identified, whilean item is a set of fragments that seem to originate from the same piece of litter

- For the plastic litter categories only the total number of plastic fragments per colour category, with specifics as follow:
    - Total number of white-transparent plastic fragments;
    - Total number of dark coloured plastic fragments (black, blue, dark green…);
    - Total number of light coloured plastic fragments (cream, yellow, pink, light green…).
- Analyse at least 10% of the detected plastic by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

Microlitter detection

- Examine the filter in the Petri dish under a stereomicroscope for particles resembling microplastics. Cover the filter with glass lids during observation to avoid the contamination of the sample.
- Photograph, count and record the type, colour and maximum length of microplastic particles using image analysis software and categorize microplastic particles.
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

Microlitter detection in faeces

For free-ranging faeces, samples should be dried and digested using KOH 10%, then the solution filtered and litter should be classified. The dry mass (grams, precision 0.01 g) of each category should be recorded after drying at room temperature for at least 24h or at 35 °C for 12 h.

Litter categories

Categorize marine litter according to the categories showed in Table 15-1. The categorization of the gastrointestinal tract contents and excreta 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 in most cases, particles can’t be unambiguously linked to particular objects. But where is possible, under notes in datasheets, the items should be described and assigned a litter category number using the “Joint List” developed by the TSG ML group (Fleet et al., 2021). In addition, it is important to measure and quantify also natural items (food and/or no food).

Table 15-1. *Classification of Marine Litter items plus Food remain and Natural no food remain (from INDICIT 2018).*

BIOTA categories for contents of digestive tract			
PLA	**PLASTIC**	**acronym**	all plastic or synthetic items: note number of particles and dry mass for each category
IND	**pellets**	**ind**	industrial plastic granules (usually cylindrical but also oval spherical or cubical shapes exist)
	probab ind?	**pind**	suspected industrial, used for tiny spheres (glassy, milky, ...) (= microbeads)
USE	**sheet**	**she**	remains of sheet, eg from bags, cling-foil, agricultural sheets, rubbish bags etc
	thread	**thr**	threadlike materials, eg pieces of nylon wire, net-fragments, woven clothing; includes 'balls' of compacted material
	foam	**foam**	all foamed plastics, polystyrene foam, foamed soft rubber (as in matrass filling), PUR used in construction etc
	fragments	**frag**	fragments, broken pieces of thicker type plastics, can be bit flexible, but not like sheetlike materials
	other	**Poth**	any other, incl elastics, dense rubber, cigarette-filters, balloon-pieces, softairgun bullets, objects etc. DESCRIBE!!
RUB	**OTHER RUBBISH**	**acronym**	any other nonsynthetic consumer wastes: note number of particles and (in principle) dry mass for each category
RUB	**paper**	**pap**	newspaper, packaging, cardboard, includes multilayered material (eg Tetrapack pieces) and aluminium foil
	kitchenfood	**kit**	human food remains (galley wastes) like onion, beans, chickenbones, bacon, seeds of tomatoes, grapes, peppers, melon etc
	other rubbish	**rubvar**	other various rubbish, like processed wood, pieces of metal, metal air-gun bullets; leadshot, paintchips. DESCRIBE
	FISHHOOK	**hook**	fishing hook remains (NOT FOR HOOKS ON WHICH LONGLINE VICTIMS WERE CAUGHT - THOSE UNDER NOTES)
POL	**POLLUTANTS (INDUS/CHEM WASTE)**	**acronym**	other non-synthetic industrial or shipping wastes (number of items and mass per category (wet 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=0.0001g if other particles smeared with tar but cannot be sampled separately)
	paraf/chem	**chem**	lumps or soft mush of unclear paraffin, wax like substances (NOT stomach oil!); if needed estimate mass by subsampling
	featherlump	**confea**	lump of feathers from excessive preening of fouled feathers (n=1 with drymass) (NOT for few normal own feathers)
FOO	**NATURAL FOOD**	**foo**	various categories, depends on the species studied, and aims of study
NFO	**NATURAL NON FOOD**	**nfo**	anything natural, but which cannot be considered as normal nutritious FOOD for the individual

Collection of data

For each organism, an assessment is made of:

1. Frequency of occurrence (%) of ingested macro and microlitter for each species, calculated as the percentage of the individuals examined with ingested macro- and microplastics.
2. Abundance (N) of macro and microlitter ingested per individual (average number of items/individual) for each species, calculated as a total and per category. Since currently there are inconsistencies in the literature in reporting abundance of ingested litter, it is recommended to report average number of items per individual considering both all individuals examined and only individuals found with ingested macro and litter.
3. Total dry weight (g) of the detected waste expressed on grams (precision: second decimal place). This weight refers to each single category found in a specific organ (or faeces) of the specimen.

Other information as colour of items, polymer of the different items (at least 10% of the total items) and different incidence of litter in oesophagus, stomach and intestine, incidence and abundance are useful for research and impact analysis.

15.6 Analysis of plastic tracers and PBTs

Plastic additives

The compounds to be detected are:

- *Phthalates:* a group of chemicals widely used as additives to make plastics more flexible and harder to break; they can interfere with endocrine system (Baini et al., 2018).
- *Bisphenol A*: used in the production of polycarbonate, can have endocrine disrupting effects (Crain et al., 2007; Halden, 2010; Oehlmann et al., 2009) and the styrene and polyvinyl chloride monomer, used in the production of polystyrene and polyvinyl chloride (PVC), can be carcinogenic and/or mutagenic (Lithner et al., 2011; Papaleo et al., 2011; Xu et al., 2004).
- *Polybrominated diphenyl ethers:* they belong to the group of brominated flame retardants (BFRs), which are used in various polymeric materials such as plastic parts, resins, textiles, and other substrates to reduce their fire hazards (BSEF 2003; Król et al. 2012).

Persistent, bioaccumulative and toxic substances (PBTs)

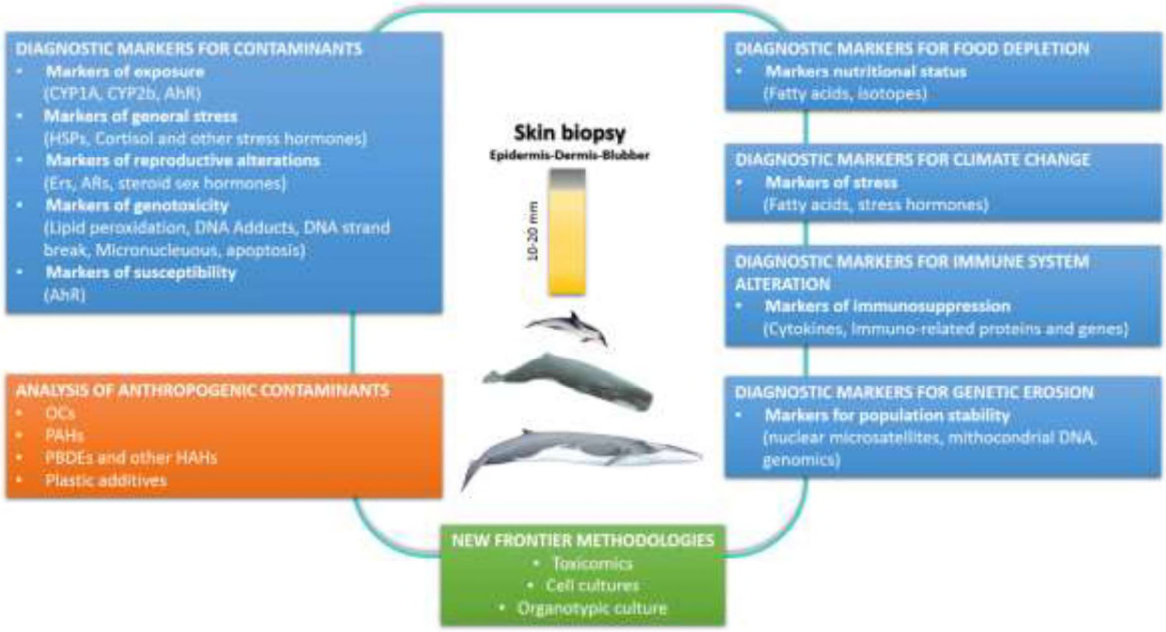
In addition to the plastic additives that may leach from plastics when released into the marine environment, plastics also tend to adsorb in their surface persistent bioaccumulative and toxic substances (PBTs) (e.g. organochlorine compounds OCs, PAHs and PBDEs) and metals (e.g., lead, copper and cadmium) that are present in the seawater.

Depending on the compounds and the tissue to be analysed, different methods should be applied to detect the presence of plastic related contaminants in the sentinel species (Annex VI).

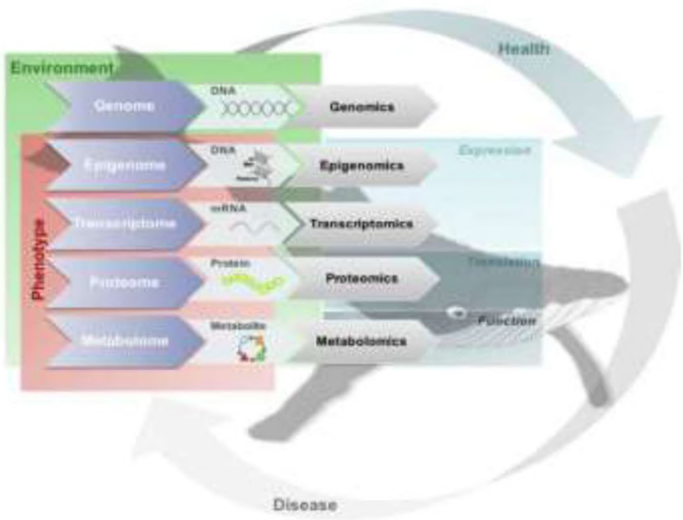
15.7 Biomarkers analysis

The toxicological effects associated with the presence of marine litter can be evaluated using a set of diagnostic and prognostic methodologies, by means of biomarkers. A non-exhaustive list of existing biomarker approaches and plastic tracers' contaminants that are usually applied in marine mammal analyses is reported in Annex VI.

Biomarkers have been selected on the basis of the level of biological responses and in relation to the main effects related to marine litter/microplastics ingestion. The selected biomarkers can diagnose different impacts related to: a) physical damages/effects of marine litter, b) exposure to/effect of chemical tracers, and c) exposure to/effect of adsorbed chemicals.



A



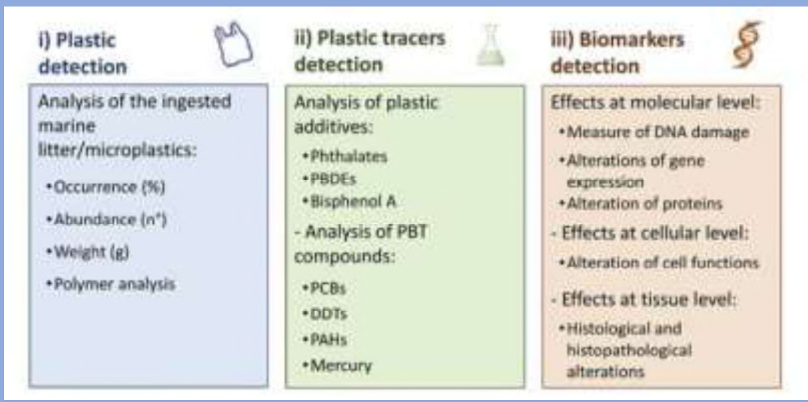
B

***Figure 15-8. A**) three-fold approach to detect the marine litter presence and impacts to marine mammals. **B**) Omics techniques in skin biopsies (from Mancia 2018).*

Starting from this initial list and building on the findings of the testing phase of the Plastic Busters MPAs project, the most suitable diagnostic tools to detect the presence and impact of ML on marine mammals are proposed here below.

Table 15-2. *Main diagnostic tools selected in the PLASTIC BUSTERS MPAs project to detect the presence and impact of ML in marine mammals.*

MARINE MAMMALS: MAIN DIAGNOSTIC TOOLS SELECTED IN THE PLASTIC BUSTERS MPAs PROJECT



1. Analysis of gastrointestinal content: litter analysis and classification (stranded organisms)
2. Analysis of faeces: litter analysis and classification
3. Analysis of plastic tracers: phthalates
4. Analysis of biological end-points: gene expression (*adipoq, ahr, gr, ppara, pparg, thra, thrb, cd36, cyp1a, cyp3a a*), Omics

15.8 Materials & Equipment for sampling

The following material and equipment are necessary for the correct application of the protocol (**stranded organisms**):

- Boots
- Camera
- Clamps (at least 6) or roast wire
- Clips with claws
- Containers for samples (Bottle/zipped bags)
- Cooler
- Cut-resistant gloves
- Garbage bag
- Glasses and protective mask or shield
- Nitrile Gloves
- Integral protective suit
- Measuring cylinders (2 L, 1L, 50cL; precision 0.1L)
- Measuring decimetre

- Measuring tape
- Metal containers
- Metal spoon
- Observation sheet
- Pen
- Permanent marker
- Precision balance
- Rope (to mark-off the zone)
- Sampling sheets
- Scalpel
- Scissors
- Sieve with 1 mm mesh
- Sieve with 5 mm mesh
- Transport bins or containers

The following material and equipment are necessary for the correct application of the protocol (free-ranging organisms):

- Aluminium foil
- Aluminium Pole
- Bicoculars
- Camera
- Crossobow
- Cryoboxes
- Cryovials
- Darts
- DMSO (20% saturated with NaCl)
- Eppendorf (0.5 ml. 1.5 ml. 2.0 ml)
- Ethanol (70%, 100%)
- Falcon tubes
- Glass Petri dishes
- Gloves
- GPS
- Liquid nitrogen dewar (in alternate dry ice)
- Net (for faces collection)
- Paper and block-notes
- Paper towels
- Pasteurs
- Pencils
- Permanent markers
- Plastic Sealable bags
- RNAlater
- Ruler
- Scalpels
- Spare batteries
- Spare camera batteries and memories
- Thermic bags
- Tips (for crossbow and aluminium pole)

- Tweezers
- VHF Radio

References

IPA-Adriatic NETCET, 2015. Standard protocol for post-mortem examination on cetaceans

Joint ACCOBAMS and ASCOBANS document "Best practice on cetacean post mortem investigation and tissue sampling" (Lonneke L. IJsseldijk, Andrew C. Brownlow, Sandro Mazzariol, 2019).

Guidance on Monitoring of Marine Litter in European Seas, 2022. A guidance document within the Common Implementation Strategy for the marine Strategy Framework Directive - Marine Litter Impact on Biota. MSFD Technical Group on Marine Litter -draft.

Fossi, M.C, Vlachogianni, T., Anastasopoulou, A., Alomar, C., Baini, M., Caliani, I., Campani, T., Casini, S., Consoli, P., Cillari T., D'Alessandro, M., Deudero, S., Galgani, Galli M., F., Kaberi H., Panti, C., Pedà, C., E. Romeo, T., Scotti, G., Tsangaris, C., Zeri, C., 2019. Toolkit for monitoring marine litter and its impacts on biodiversity in Mediterranean MPAs. Interreg Med Plastic Busters MPAs project (D.3.3.2).

Monitoring Marine Litter (Macro-Micro) in biota: stranded marine mammals

Species:		**ID code:**	
Location/Country:		**Latitude**	**Longitude**
Discovery circumstances	☐ By catch/Fishery ☐ Found at sea ☐ Stranding ☐ Dead at rescue center ☐ Other		
Discovery circumstances (*Please specify according to the toolkit*)			
Date of discovery			
Date of necroscopy			

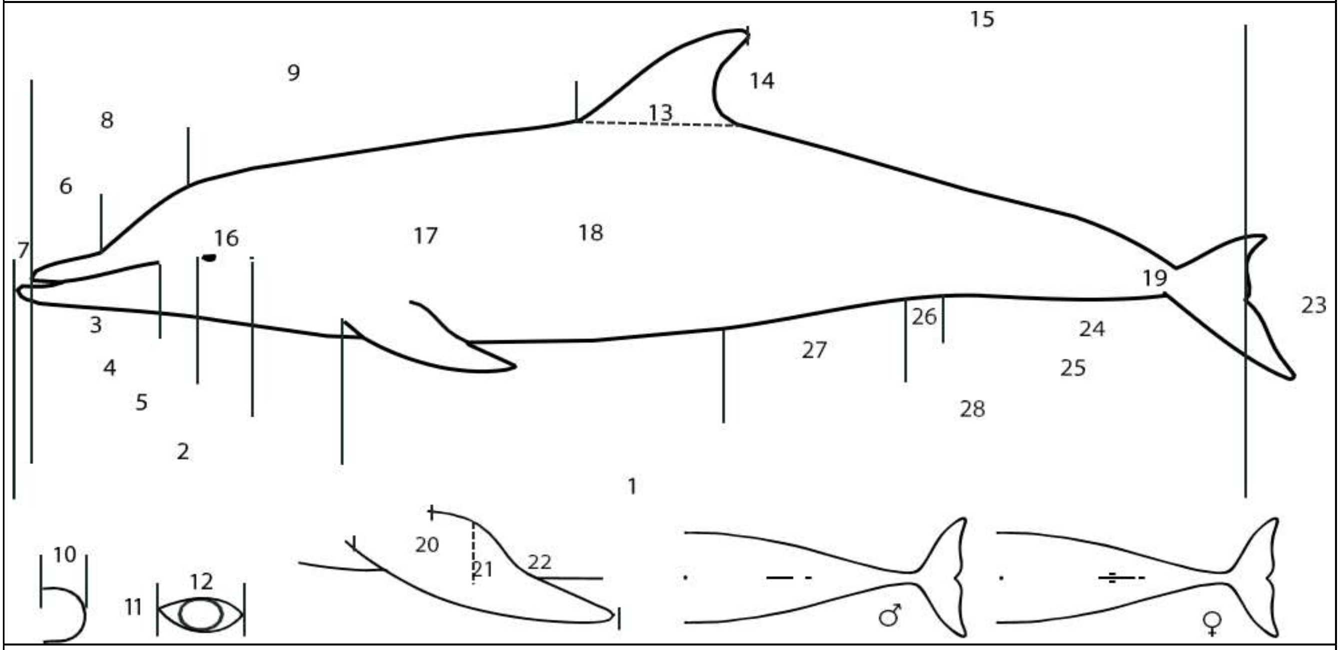
Animal body condition				
Conservation status	☐ Level 1	☐ Level 2	☐ Level 3	☐ Level 4
Health status	☐ Poor	☐ Fair	☐ Good	
Main injuries	☐ No injuries ☐ Abrasion	☐ Fracture	☐ Amputation	☐ Sectioning
Affected parts	☐ Wings	☐Tail	☐Neck	☐ Head ☐ Other
Fat reserve	☐ Thin	☐ Fat	☐ Normal	☐ Not recorded (NR)

Collected tissues		**N°. of aliquots**
Muscle		
GI tract		
Liver		
Fat tissue		

Necropsy performed by:

Name and Institution:

Biometric measurement



Sex: ☐ Male ☐ Female ☐ Not identified

1. Total length (cm):	15. Dorsal fin flukes (cm):
2. Snout anterior insertion or flipper length (cm):	16. Body height at eye (cm):
3. Snout angle of gape length (cm):	17. Body height at posterior insertion of flipper (cm):
4. Snout eye length (cm):	18. Body height at interior insertion of flipper (cm):
5. Snout ear length (cm):	19. Caudal peduncle circumference (cm):
6. Rostrum length (cm):	20. Flipper maximum width (cm):
7. Snout rostrum length (cm):	21. Flipper length (at anterior insertion) (cm):
8. Snout blowhole length (cm):	22. Flipper length (at posterior insertion) (cm):
9. Snout dorsal fin length (cm):	23. Flukes width (cm):
10. Blowhole length (cm):	24. Anus – flukes length (cm):
11. Blowhole width (cm):	25. Genital aperture flukes (cm):
12. Eye’s diameter (cm):	26. Anus – genital aperture length (cm):
13. Dorsal fin length (cm):	27. Umblicus – genital aperture length (cm):
14. Dorsal fin height (cm):	28. Umblicus flukes (cm):

Notes and remarks:

Monitoring Marine Litter (Macro-Micro) in biota: free-ranging cetaceans

Species:			**ID code:**		
Sampling date and time:					
Sampling site:			**Latitude**		**Longitude**
Sampling tool:	☐ Pole			☐ Crossbow	
☐ Single	☐ Couple	☐ Mother + Calf	☐ Group	Dimension of the group:	
Picture ref.:					
Weather condition		**Sea:**		**Wind:**	
Lenght (m):					
Aprox. age:	☐ Adult		☐ Sub-adult		☐ Juvenile
Side sample and position:	☐ Right		☐ Left		

Aliquots	**Contaminants (blubber)**	**Liquid N_2**	**RNA later**	**DMSO**	**Cell medium**	**Bouin**
	PCR (skin)					
	WB (skin)					
	Sex (skin)					
	Isotopes (skin)					
	Omics					
	Histology					
Slices	**Treatment**	**Dose**	**Time**	**Hour-notes**		
Faeces						

Notes and remarks:

ANNEX VI

Table A-9. *Tissues and methods to be used to detect plastic tracers in marine mammals.*

	CHEMICAL COMPOUND	TISSUE/SAMPLE	ANALYTICAL METHOD
PLASTIC ADDITIVES	**Phthalates**	Blubber, muscle, liver, whole organism, skin biopsy	Baini et al., (2017), Fossi et al., (2016), Savoca et al., (2018), Routti et al., (2021)
	Bisphenol A	Muscle	Ballesteros-Gómez et al., (2009)
		Blubber, skin biopsy	Xue et al., (2016)
		Blood	Cobellis et al., (2009)
	Polybrominated diphenyl ethers	Blubber, muscle, liver, blood, skin biopsy	Muñoz-Arnanz et al., (2016), (Zaccaroni et al., (2018), Bartalini et al., (2019), Baini et al., (2020), Aznar-Alemany et al., (2021)
ADSORBED CONTAMINANTS	**Polycyclic aromatic hydrocarbons**	Blubber, muscle, liver, blood, skin biopsy	Marsili et al., (2001)
	Organochlorine contaminants	Blubber, muscle, liver, blood, skin biopsy	Marsili and Focardi, (1997), Bartalini et al., (2019), (Genov et al., 2019), Baini et al., (2020), Aznar-Alemany et al., (2021)
	Mercury	Blood, skin, skin biopsy	Correa et al., (2013)

Table A-10. *Biological end point detected in marine mammals by the biomarker and Omics approach.*

EFFECT	TISSUE	TEST
GENOTOXICITY	Blood	Comet assay (Molino et al., 2019) (*) Mn test (Bolognesi et al., 2006) ENA assay (Casini et al., 2018); (Pacheco and Santos, 1997)
OXIDATIVE STRESS	Liver, kidney	CAT, GST, LPO, GPX, GR, GSH (Yu et al., 2018) (*)
	Plasma, skin	LPO (Fossi et al., 2016), Casini et al., 2018) CAT (Fossi et al., 2013)
IMMUNOTOXICITY	Blood	Total and differential white blood cells (WBC) count (Casal and Orós, 2007; Davis et al., 2008; Caliani et al., 2019) H:L ratio (Caliani et al., 2019) Respiratory burst (Secombes, 1990; Caliani et al., 2019) TAS assay (Miller et al., 1993; Caliani et al., 2019) Lisozyme enzyme (Keller et al., 2006; Caliani et al., 2019) casp8, casp9, TRAF (Karami et al. 2017; Mathieu-Denoncourt et al., 2015) (*)
REPRODUCTION	Plasma, Gonads	CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015) (*) Vitellogenin (Fossi et al., 2004)
	Plasma, skin	Vitellogenin (Herbst et al., 2003) CYP17A, CYP19, ERs, VTG, StAR (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*)
HISTOPATHOLOGY INFLAMMATION AND MORPHOLOGY	Liver, kidney	Histopathology, histology (Pedà et al. 2016; Karami et al. 2017; Batel et al., 2018) (*)
XENOBIOTIC METABOLISM AND BIOTRANSFORMATION	Blood, skin, feces, liver	CYP1A; AHR, CYP3A (Fossi et al. 2014, Panti et al. 2011; Rochman et al., 2013) (*) Porphyrins (Guerranti et al., 2014) (*)
NEUROTOXICITY	Brain, muscle, plasma	AChE, BChE (Barboza et al., 2018) (*)

CELLULAR STRESS	Blood, skin, liver, kidney	PPARA, PPARG, HSP70, GPX, E2F1 (Mathieu-Denoncourt et al., 2015; Panti et al., 2011) (*) Gamma glutamyl transferase (GGT) (Nematdoost Haghi and Banaee, 2017) (*) Cortisol and corticosterone (Flower et al., 2015) LDH (Nematdoost Haghi and Banaee, 2017) (*)
OMICS	skin	Epigenetics (Mancia et al., 2021), Transcriptomics (Lunardi et al., 2016)

(*) effects detected after laboratory or field exposure with MPs or Plastic Tracers.