Guidelines for monitoring microlitter in biota of the Baltic Sea: Bivalves

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1. Introduction

Marine Litter and Microlitter are defined according to Commission Decision 2017/848 (2017) and UNEP (2022): “Marine litter is any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment” (UNEP, 2022)”.

Marine microlitter is marine litter with a length of its maximum dimension below 5 mm (Galgani et al. 2023).

The scope of microlitter monitoring in biota within HELCOM is in accordance with MSFD Com Dec “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” (Commission Decision (EU) 2017/848, 2017).

2. Sampling of mussels for microlitter monitoring in biota

2.1 Species selection

According to the Commission Decision "litter and micro-litter should be assessed, where possible, in representative species from the following groups: birds, reptiles, fish, or invertebrates. Fish and invertebrates are particularly relevant species for the assessment of ingested micro-litter" (European Commission 2022: 79). Within these draft guidelines we focus on bivalve species representing invertebrate at this first stage. Compared to fish species the investigation of invertebrates has several advantages in terms of

- Spatial representativeness (widespread occurring in coastal waters and close to pollution sources, representing a more stationary habitat, filtering large volumes of water and/or sediment)
- Temporal representativeness (reflecting a time integrated exposure window)
- Feasibility (relatively easy to sample, easy to be used in cage studies, easily to be sampled in parallel to other monitoring programmes)

Following the guidance on the monitoring of marine litter in European Seas published by the European Commission (Galgani et al. 2023) the monitoring of microlitter in invertebrates targets on mussel species. Based on the habitat preferences and thus occurrence of species in HELCOM regions *Mytilus spp.* (L.), *Macoma balthica* (L.) and *Dreissena spp.* (L.) are proposed as potential indicator species.

2.2 Sampling conditions

2.2.1 Number and location of monitoring stations

The number of monitoring stations surveyed by each country depends on the heterogeneity across stations and areas as well as on how many sub-basins each respective country encompasses. The distribution of monitoring stations should represent variation within HELCOM sub-basins and should, where possible, integrate stations for target and measure monitoring[1] (i.e. near coast locations that are related to potential point-sources or locations of potential accumulation areas) as well as state monitoring[2] (i.e. open water or offshore-locations) according to the technical guidance on monitoring for the Marine Strategy Framework Directive (Zampoukas et al. 2014).

It is recommended to select stations for monitoring of microlitter corresponding to existing monitoring stations from other monitoring programmes such as the monitoring of biodiversity components and/or contaminants.

[1] "Target and measure monitoring (relating to Art. 10 and 13 MSFD) which compares to WFD operational monitoring: This requires additional monitoring (in terms of indicators/parameters, sampling frequency and stations) in those areas and for those ecosystem components for which GES has been failed and for those pressures, which are responsible for failing GES and for which environmental targets have been set. Monitoring should enable to assess progress towards GES and achieving targets and the efficiency of measures." (Zampoukas et al. 2014: 15).

[2] State monitoring (relating to Art. 8, 9 MSFD) which compares to WFD surveillance monitoring: It aims at long-term monitoring and at surveillance monitoring for an overview of the state of the environment and is the backbone of MSFD monitoring. It is sufficient where GES is achieved for the individual ecosystem component. State monitoring includes the features, activities and pressures relevant for GES. It includes monitoring of additional parameters under Annex III MSFD to assess the extent and intensity of human activities and resulting pressures and their changes as well as changes in natural conditions." (Zampoukas et al. 2014: 15).

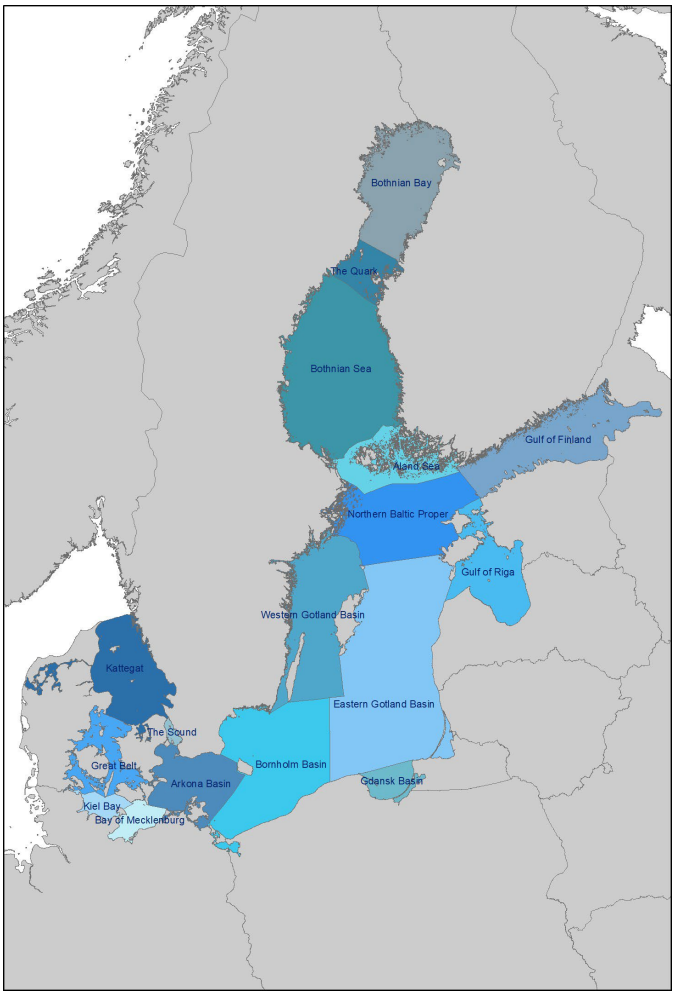

Figure 1. Map of the Baltic Sea presenting the HELCOM sub-division into 17 open sea sub-basins (HELCOM 2022).

2.2.2 Frequencies and time of sampling

The suggested frequency of monitoring for microlitter in mussels at stations selected for temporal trend monitoring is once per year at the same time and outside of the spawning season, preferably in late summer. The monitoring frequency at stations for target and measure monitoring might be conducted at lower frequencies.

The sampling time depends on feasibility and whether the sampling is carried out in accordance with other monitoring programmes that require a specific season for sampling.

2.3 Sampling techniques

2.3.1 Sampling strategy, sample numbers, replicates

Sampling of mussels can be done from natural populations i.e. in parallel with other monitoring programmes such as the macrozoobenthos monitoring or if feasible in terms of the selected species by a caging strategy. The sampling technique for natural populations depends on the specific habitat of the selected species and might vary from grab sampling to manual collection. For these samples, a minimum of 3 alive individuals should be taken depending on the targeted sample volume to be analysed in the laboratory. These should refer from the same size class. It is further recommended to avoid taking individuals growing on or close to any synthetic substrate. A replicate is encouraged.

For the caging strategy, mussels are recommended to be collected from a clean site with similar environmental conditions as the planned caging area. 50 mussels are placed in one cage. Before cage deployment, the mussels should be kept in aerated water from the collection site. The number of individuals must include the cage mussels (30-50) and the individuals (20 ind.) to determine the start situation/condition of the natural population.

The cages should include boxes, bags or equivalent structures, where the mussels are placed. Mesh-like structure is recommended to allow water to enter the box without mussels dropping out from the cage. Cages manufactured of metal (AISI 316 stainless steel) are recommended. The cages are anchored to the bottom with a rope attached to a weight and held in a stable vertical position by submerged buoys at 4-10 m depth. The caging should be carried out after breeding season (in August – September) for 3-4 weeks in the natural geographical habitat of the mussels. In salinity >5 Mytilus spp. should be preferred.

After the caging period the mussels are retrieved and 20 healthy individuals are collected for microlitter ingestion determination. 5 mussels, preferably same size, are pooled as one sample, 20 individuals producing 4 parallel samples. The mussels should be dissected immediately after every sampling or frozen and processed later.

2.3.2 Recording of basic parameters, sampling protocol

Basic parameters during sampling shall be recorded and include:

a) Mandatory: date and time, cruise ID, sampling platform, station name and internal identification code (ID), station classification (water and land station type and monitoring station type), coordinates, waterdepth, species, sampling strategy (natural populations or caging strategy), start and end of deployment (caging strategy).

b) Optional: weather and sea conditions, substrate.

Sample codes and parameters are documented in the sample documentation form. The respective sample containers are labelled with (at least): date, station code, station name and the internal code for laboratory processing (ID).

2.3.3 Sample transportation, preservation and storage

Individuals are wrapped in aluminium foil and pooled into one composite sample per replicate and preferably immediately frozen at -18 °C. The use of plastic bags or containers is to be avoided as much as possible. The specific storing conditions depend on the storage time and conditions during the sampling campaign and/or on the schedule of the laboratory analyses.

2.4 Sampling QA/QC

To minimise background contamination, the following measures should be considered within the sampling campaigns, also when they are carried out in parallel to other monitoring campaigns:

a) Use of glass or aluminium/metal materials only, avoid the use of synthetic materials.

b) Pre-cleaning of sample containers and instruments with filtered water and/or ethanol or isopropanol. Glassware can also be subject to baking within a muffle oven at 500 °C.

c) Operators to avoid wearing synthetic clothes. Operators to position facing the wind while retrieving the sample. Operators to take care that potential contamination sources during sampling and sample processing are avoided.

d) Integration of blank samples: a representative number of blank samples are integrated to account for contamination during sampling. The number of blank samples should be preferably 10 % of the total number of samples (at least 3).

e) The total number of blank samples should be representative for varying sampling conditions during the respective sampling campaign and thus, should reflect the specific contamination potential e.g. through varying weather conditions, varying operators wearing varying clothes.

f) It is recommended to retrieve material from any device of synthetic polymer origin implemented during sampling. These comparative materials should be investigated for their polymer composition to enable exclusion of clearly identified contamination from sampling devices.

g) For generating blank samples, an empty sampling container is positioned next to the sample and opened while retrieving the sample. The resulting blank sample is subject to laboratory analyses in the same manner as mussel samples.

3. Sample treatment / laboratory analyses

Sample treatment and laboratory analyses can be done applying different methods when specific quality criteria are ensured. Any sample treatment needs to ensure not to harm synthetic polymers by applying strong chemicals and high temperatures. The treatment process and methods applied need to be controlled via contamination control and recovery tests with reference samples.

3.1 Laboratory QA/QC

3.1.1 Contamination control

Appropriate measures to reduce air contamination, cross-contamination and contamination control must be taken during laboratory analysis. These include:

- Wearing of personal protection equipment made of natural materials (cotton laboratory coats, avoid plastic fibre face masks).
- Ensuring clean laboratory conditions (regular cleaning, regulated air circulation, minimized presence of staff, use of clean room and laminar flow chambers combined with fume hoods if possible).
- Avoidance of any plastic materials during analyses (preferred use of glass and stainless steel materials).
- Pre-filtration of water and chemical solutions with filter pore size significantly lower than minimal cut-off size of targeted particles in the samples.
- Pre-cleaning of beakers and instruments.
- Pre-cleaning of filters (rinsing, annealing according to filter material).
- Covering samples and working solutions throughout the sample processing.
- Reduction of processing steps as far as possible.
- Inclusion of a relevant number of blank samples analysed in parallel with each sample series.
- Inclusion of a relevant number of reference samples analysed in parallel with each sample series.

3.1.2 Blank samples and recovery tests (mandatory)

a) A relevant number of blank samples is to be analysed in parallel with each sample series (set of samples investigated in parallel in one laboratory processing cycle). Combining field blank and laboratory blank samples is not recommended since the number of samples processed within one sample series may differ from the number of samples being representative for the respective field blank sample.

b) Microlitter particles detected within both, field and laboratory blank samples, are used to calculate the limit of detection (LOD - mean + 3 x standard deviation of the particle concentration) according to McDougall et al. (1980). LOD thus reflects the efficiency of the precautionary methods during sampling and sample processing of the respective laboratory. LOD is reported within the data. Blank values are not subtracted from the results.

c) A relevant number of reference samples is to be analysed in parallel with each sample series. Reference samples reflect the efficiency of the respective laboratory protocol and are treated in the same manner and throughout all steps as the mussel samples. Reference samples should preferably encompass real mussel samples that are spiked with a relevant number of synthetic polymer particles that are representative for dominating size categories, morphologies and polymer composition of the particles to be detected within the mussel samples. The number of added reference particles is to be discussed. The number of reference particles will affect the resolution of the recovery

rates, thus, a number of at least 50 reference particles for both fragments and fibres could be recommended leading to a resolution of 2 %.

d) The recovery ratio (%) is calculated for re-detected added reference particles as the mean value accounting for different size categories, morphologies and polymer composition. It is recommended to include reference material containing three types of polymer with different densities, three morphologies and a similar size to the targeted lower cut-off size (i.e. 50 µm) of particles according to Cui et al. (2022). The mean recovery ratio is reported together with the data. Results on mussel samples are not corrected for recovery rates.

3.2 Sample recording of dimensions and weight and sample preparation for laboratory analyses

Mussels are retrieved from aluminium foil and shell surface is rinsed with filtered water with collecting the rinsing solution. The individuals are thawed slightly and measured for their dimensions and gross weight with an analytical balance (accuracy minimum 0.01 mg). Mussel shells and the byssus are dissected from each individual and pooled samples of soft body from the individuals are weighed into pre-cleaned glass beakers. The rinsing solution derived from rinsing the individuals and the rinsing solution resulting from 3-fold rinsing per aluminium foil are also added to the beaker.

3.3 Sample digestion

In general, the order of digestion and a potential density separation depends on the sample treatment protocol and particle analysis technique of the processing laboratory.

Optional digestion protocols cover oxidative, enzymatic, alkaline or mixed treatments. The implementation of acid digestion is not recommended since especially strong acids proofed to affect synthetic polymers. The duration of the sample digestion depends on the selected digestion protocol.

The application of low temperatures and stirring of the samples are optional add-ons within sample digestion. The application of temperatures >50°C is to be avoided since it may damage synthetic polymers.

After digestion, the digestion solution is rinsed-off over a sieve with the mesh size of the recommended minimum size of targeted particles (50 µm in accordance with the EU guidance on marine litter (Galgani et al. 2023), see chapter 3.5 and 4.3 for further options).

If particle dimensions are not determined by single particle a size separation step with a sieving cascade encompassing at least 50, 100, 300 and 1000 µm can be applied at this stage (smaller mesh sizes are optional).

3.4 Density separation

The application of a density separation step is optional and depends on the amount of sediment particles present. The choice of the density solution and the device used for density separation depends on the respective protocol applied. Density solutions cover zinc chloride ($ZnCl_2$), sodium iodide (NaI), and sodium polytungstate ($NaWO_4$) with a minimum density of 1.5 g/cm³. The application of solutions with densities >1.7 g/cm³ is recommended since this will distinctly improve the recovery rates of synthetic particles of higher material densities. The use of sodium chloride (NaCl) is not recommended since a relevant number of synthetic polymers will not be recovered due to low solution density.

In general, samples are introduced into the density separation solution, stirred for 10 minutes and left for settling for 24 h. The supernatant suspension is rinsed thoroughly with filtered water and

transferred onto filters applicable for the further particle identification. Filters are left to dry in pre-cleaned glass petri dishes. It is recommended to repeat the density separation process at least once.

3.5 Particle identification

The identification of synthetic particles depends on the device available and varies between optical microscopic identification, spectroscopic approaches like FTIR and Raman spectroscopy and staining approaches like Nile red staining in combination with fluorescence microscopy. Particles are identified according to numbers, size classes, morphology, colours (optional) and polymer composition (on at least a subset).

3.6 Polymer identification

The determination of at least a subset of particles for their polymer composition via e.g. FTIR or Raman spectroscopy or mass-based techniques is mandatory. Device settings and minimum library match (%) attributed is to be recorded within the metadata. Spectra libraries utilised for polymer composition determination should integrate spectra from synthetic and organic components and weathered synthetic polymers. It is suggested to agree on one or several libraries that are used by all processing laboratories and/or to generate a combined FTIR and/or Raman spectra library for HELCOM microlitter monitoring.

It is recommended to analyse the polymer composition on a representative subset with a minimum of 10 % of synthetic particles identified within the size categories from 50 to 1000 µm. The subset size of particles identified in any smaller size category is to be discussed. The particles integrated in the subset are to be selected representatively according to size categories and morphologies.

4. Parameter and data recording

Prenote: further decisions regarding the data portal for reporting data on microlitter in biota are to be made. In any case, the parameters to be reported are listed below.

4.1 Numbers

The recording of number of particles identified as synthetic polymers/microlitter is mandatory.

Data are calculated to number of particles and optionally weight in grams per kg fresh weight of dissected soft tissue. At this stage, no recommendation on re-calculating number of particles into mass is given. The development of conversion algorithms based on polymer composition and particle size/volume is to be evaluated.

4.2 Morphology

The morphology of all identified particles is to be recorded according to the following morphology classes:

Table 1 Morphology classes to be used to report all identified particles.

NERC Vocabulary Server (NVS) identifier "microlitter morphology"	Name	Definition according to NVS	Definition according to GESAMP 2019 (Kershaw et al. 2019)
H0100004	Filaments	Slender thread-like micro-litter particles	"Line" (Fibre, filament, strand): long fibrous material that has a length substantiallylonger than its width
H0100005	Films	Micro-litter particles derivedfrom plastic sheets or thin plastic films	"Film" (sheet): flat, flexibleparticle with smooth or angular edges
H0100006	Foams	Any kind of micro-litter particle made of plastic foam,including EPS and XPS foams	"Foam" (EPS, PUR): near spherical or granular particle,which deforms readily under pressure and can be partly elastic, depending on weathering state
H0100002	Fragments	Irregularly-shaped plastic micro-litter particles with broken off edges that may berounded or angular	"Fragment" (flake): irregular shaped hard particleshaving appearance of being broken down from a larger piece of litter
H0100003	Pellets / Granules / Beads	Microlitter particles which are spherical, flat on one side or cylindrical in shape.	hard particle with spherical, smooth or granular shape

It is to be evaluated whether microbeads are to be reported as a single class or identified from the data set as morphology: granules and the (smaller) dimension in size compared to pre-production resin pellets.

It is to be evaluated whether "pellets" and "granules" should be separate classes. In addition, it has to be considered that "film" and "foam" might not be identified due to restrictions of devices or protocols especially within the smaller size fractions.

4.3 Particle dimensions

The dimensions of identified particles should be recorded according to the following size classes:

- 50 – 99 µm
- 100 – 299 µm
- 300 – 999 µm
- 1000 – 4999 µm

The reporting of size classes below 100 µm is optional according to the following size classes:

- 20 – 49 µm
- <20 µm

It is to be pointed out that results may be biased if particle dimensions are retrieved from mesh sizes from sieving and filtering or measuring of actual particle length and width dimensions.

The reporting of absolute dimensions on particle length and/or particle width is optional.Sizes

of particles are defined according to:

e) Length (maximum Ferret diameter in longitudinal orientation)

f) Width (maximum Ferret diameter perpendicular to the identified length transect)

Fibres with a length > 5000 µm are considered “mesolitter” and are therefore excluded from the data analysis.

4.4 Polymer composition

Polymer composition is to be reported according to polymer classes and is to be defined for at least a subset of identified synthetic particles.

It is suggested to align the polymer types according to the list modified from AMAP 2021 (see Table 2) but to set up a short list with prioritised synthetic polymers that are predominantly found in environmental samples and that at least have to be reported when occurring.

Table 2 Polymer types for data reporting (modified from AMAP 2021: 225)

Polymer type name	**Examples of materials included (detailed level)**
Acrylonitrile based	acrylonitrile butadiene styrene(ABS), polyacrylnitrile (PAN)
Cellulose based	cellulose acetate (CA), cellulose nitrate (CN)
Polyamide based	all types of polyamide (PA) like various nylons
Polycarbonate based	polycarbonate (PC)
Polychlorinated polymers	polyvinyl chloride (PVC), chlorinated PE, various chlorinated polymers
Polyester based	polyethylene terephthalate (PET), all other types of polyesters
Polyethylene based	high density polyethylene (HDPE),low density polyethylene (LDPE), and copolymers with a major PE fraction including ethylene-vinyl acetate copolymer (EVA)
Polyfluorinated polymers	polytetrafluoroethylene (PTFE)
Polymeth(ester)acrylate based	all types of polymeth(ester)acrylate (PM(ester)A)
Polypropylene based	polypropylene (PP), and copolymers with a major PP fraction
Polystyrene based	polystyrene (PS), and copolymers with a major PS fraction
Polyurethane based	all types of polyurethane (PUR)
Varnish/paint particles	If different from PM(ester)A
Other plastics	polyether ether ketone (PEEK), polyoxymethylene (POM), polyvinyl acetate (PVA), polylactic acid (PLA), polyhydroxyalkanoate (PHA)
Other semi-synthetic polymers	rayon
Rubbers, automotive	styrene butadiene rubber (SBR), tyre wear
Other rubbers	ethylene propylene diene monomerrubber (EPDM), silicone, nitrile rubbers and natural rubbers
Other microlitter materials	metal, glass

4.5 Optional parameters

The recording of particle colours and/or transparency is optional. Colours and transparency are classified according to EMODnet:

Colour classes:

- black
- grey
- white (including creams)
- red
- orange
- yellow
- green
- blue (including cyan)
- purple (including biolets)
- pink (including magentas)
- brown (including tans)
- multicolour (particles made up of two or more different colours)
- colourless (particles without added dyes, pigments and/or other additives (e.g. carbon black in rubbers). This class also excludes particles that get natural colours during their production (e.g. ABS gets a natural pale-yellow colour during production))

Transparency:

- opaque (term that qualifies microlitter particles that do not allow the light to travel through them)
- transparent / translucent (term that qualifies microlitter particles that allow light to travel through them either almost unaltered (transparent) or with some diffraction (translucent, translucid or semi-transparent))

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