**TITLE:**

**Protocol for microplastics sampling on the sea surface and sample analysis**

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**SHORT ABSTRACT:**

The protocol below describes the methodology for: microplastics sampling on the sea surface, separation of microplastic and chemical identification of particles. This protocol is in line with the recommendations for microplastics monitoring published by the MSFD Technical Subgroup on Marine Litter.

**LONG ABSTRACT:**

Microplastic pollution in the marine environment is a scientific topic that has received increasing attention over the last decade. The majority of scientific publications address microplastic pollution of the sea surface. The protocol below describes the methodology for sampling, sample preparation, separation and chemical identification of microplastic particles. A manta net fixed on an »A frame« attached to the side of the vessel was used for sampling. Microplastic particles caught in the cod end of the net were separated from samples by visual identification and use of stereomicroscopes. Particles were analyzed for their size using an image analysis program and for their chemical structure using ATR-FTIR and micro FTIR spectroscopy. The described protocol is in line with recommendations for microplastics monitoring published by the Marine Strategy Framework Directive (MSFD) Technical Subgroup on Marine Litter. This written protocol with video guide will support the work of researchers that deal with microplastics monitoring all over the world.

**INTRODUCTION:**

Microplastic pollution in the sea represents a growing concern to contemporary society, due to the constant increase in plastic production and its subsequent disposal and accumulation in the marine environment1. Even if plastic macro litter would no longer enter the seas, microplastic pollution would continue to grow due to fragmentation of already existing plastic litter in the sea2. The majority of microplastic pollution studies were carried out in marine and fresh water ecosystems and mainly addressed sea surface pollution3.

The term microplastic refers to plastic particles smaller than 5 mm in size4. This term describes a heterogeneous mixture of particles, which can differ in size (from a few microns to several millimeters), color and shape (from very different shapes of fragments to long fibers). Microplastic particles can be of a primary or secondary origin5. Microplastic of primary origin is manufactured as small particles used in the cosmetics industry (pilling crème etc.) or chemical industry as precursor for other plastic products (e.g. plastic pellets used in plastic industry). Microplastic of secondary origin arise via the degradation of larger plastic pieces in the environment due to physical and chemical processes, induced by light, heat, oxygen, water and organisms6. In 2015, four types of microplastic sources were defined: larger plastic litter, cleaning products, medicines and textiles6. The main source (80 %) of larger plastic litter is assumed to be land based7. Microplastic from cosmetic products, medicines and textile enters water ecosystems through sewage and storm waters6. Microplastic particles most frequently found in water ecosystems are fragments from larger plastic litter and textile fibers8.

Microplastics have several negative effects on the environment. Their small size allows them to enter the food web through ingestion by marine organisms9, 10. Ingested particles can cause physical damage or block the digestive system of animals11. Particles can also be carriers of persistent organic pollutants (POPs). Their hydrophobic surface and favorable ratio of large surface area to small volume, enables POPs to adsorb onto the microplastics12. In the environment or digestive systems of animals who ingest them, POPs and other plastic additives can be leached from microplastic particles13.

Previous studies reported the ubiquitous presence of microplastics in the marine environment3, from the water column to the bottom sediments. The threat of microplastic pollution was already identified by the Marine Strategy Framework Directive in the EU and, consequently, mandatory monitoring of microplastics was advised14. Accordingly, the EU Technical Subgroup on Marine Litter (TSG-ML) prepared recommendations for monitoring of microplastics in the European seas15. Thus, the video guidelines for microplastics sampling are of high importance, as they support comparative monitoring and a coherent management process all over the world.

This protocol was developed within the DeFishGear project for the first monitoring of microplastic pollution in the Adriatic Sea. Recommendations from the document “Guidance on Monitoring of Marine Litter in European Seas” by TSG-ML15 were taken into account. This protocol describes the methodology for microplastics sampling on the sea surface, separation of microplastics from the samples, and chemical analysis of microplastic particles to confirm that particles are from plastic material and to identify the type of plastic. Sampling was done by the use of a manta net, which is the most suitable equipment for sampling in calm waters16. Separation of microplastics from the samples was carried out by visual identification using a stereomicroscope. Isolated particles were later chemically identified using Fourier transform infrared (FTIR) spectroscopy and micro FTIR spectroscopy.

**PROTOCOL:**

Sampling of microplastics on the sea surface

* 1. Deploy the manta net from the side of the vessel using a spinnaker boom or »A-frame« using lines and karabiners.
  2. Deploy the manta net out of the wake zone (approx. 3 - 4 m distance from the boat) in order to prevent collecting water affected by turbulence inside the wake zone.
  3. Write down the initial GPS coordinates and initial time in the data sheet.
  4. Start to move in one straight direction with a speed of approx. 2 - 3 knots for 30 min and begin the time measurement.
  5. After 30 min stop the boat and write down final GPS coordinates, the length of the route (the most correct way is to calculate the length from the GPS coordinates) and the average boat speed into the data sheet provided and lift the manta net out of the water.
  6. Rinse the manta net thoroughly from the outside of the net with seawater using a submersible pump or water from the boat water reservoir. Rinse in the direction from the manta mouth to the cod end in order to concentrate all particles adhered to the net into the cod end.

Note: never rinse the sample through the opening of the net in order to prevent contamination.

* 1. Safely remove the cod end and sieve the sample in the cod end through a 300 µm mesh size sieve or less.
  2. Rinse the cod end thoroughly from the outside and pour the rest of the sample through the sieve. Repeat this step until there are no longer any particles inside the cod end.
  3. Concentrate all material on the sieve in one part of the sieve.
  4. With the use of a funnel, rinse the sieve into a glass jar or plastic bottle using 70 % ethanol.
  5. Close the bottle; wipe it with paper towels and label the lid and outside of the jar with the sample name and date with waterproof marker (you should also put a second label written with a pencil on velum paper in a jar to avoid the possible loss of the sample name due to the erased label on the jar). Transfer labeled plastic bottle into the cool box.

Note togeneral sampling conditions: The wind speed should not be more than 2 Beaufort, since the waves are too high and the net is not stable on the sea surface. It is important to maintain a steady linear course at a constant speed during the trawls.Half of the manta net opening should be submersed during sampling.Duration of sampling should be 30 min (in cases where there is a large amount of natural material, e.g. plankton bloom, the duration of sampling can be shorter).Avoid the use of plastic tools and containers. Avoid synthetic clothing (e.g. fleece), ropes and contact of manta net with vessel to prevent contamination of the sample. Be very careful not to damage the manta net or the boat hull while deploying and capturing the net.

Separation of microplastics from the sea surface samples

## If the sample does not contain any items larger than 25 mm and appears to be clean, continue directly with step 2.3.

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## Pour sample through the sieve (≤300 µm mesh size) and remove all natural or artificial litter objects of a size >5 mm (macro and mezzo litter) from the sample, using visual identification and tweezers. Be careful to rinse each removed object carefully with distilled water in order to remove any microplastic litter adhered to it. Store all natural and artificial litter objects in separate containers. Dry all natural and artificial litter objects in a desiccator (or in the open air, but in a closed dish) and weigh them. Identify all litter objects >25 mm (macro litter) according to the Master List of Categories of Litter Items16.

## After removing all larger objects, concentrate all remaining pieces in one part of the sieve using squirt bottles or tap water. Pour the sample into a glass container using a minimum amount of 70 % ethanol with the help of a funnel.

Note: In this step the use of 70 % ethanol is crucial to preserve the sample. Also in the step of visual inspection of the sample, ethanol helps to discolor the organisms and colorful plastics therefore become easier to find.

## Take a small amount of the sample (subsample) and pour it into a glass Petri dish. Analyze the sample with the use of a stereomicroscope (20 - 80x zoom) and search for microplastic particles.

## Each microplastic particle should be categorized into one of the categories listed in Table 1 and put into a Petri dish or other glass vials, marked with a category name. The Petri dish needs to be closed at all times.

## Note: When separating microplastics from your sample be conservative and select more rather than less particles for the analysis. The real chemical structure of particles will still be determined later on. Be sure to analyze larger objects from all sides as microplastics may be stuck and therefore hidden under larger items. It may also be helpful to move already analyzed objects to one side of the Petri dish.

## Put the Petri dish under the microscope with measuring equipment (ocular ruler calibrated by the micrometer slide or image analysis software) and measure the size of each particle (measure the longest diagonal), except filaments, and note its color. Each subsample should be reviewed by another person. Be careful to rinse the glass container containing the sample so that all particles adhering to the glass walls are washed into the Petri dish.

## Weigh the microplastic particles of each category separately by the use of analytical scale. Microplastic particles need to be dried prior to weighing. The closed Petri dish can be put in a desiccator or the samples can be left to dry in a closed dish till particles became dry (the weight of closed petri dish with particles is constant).

2.9) Identify micro litter.

2.9.1) When analyzing a sample in search of microplastics, please consider that some particles will be easily visible (color, shape, size) while others may be trickier to find. Below are a few features that identify microplastic particles in the sample: For example, no cell structure, uneven, sharp, crooked edges, uniform thickness, distinctive colors (blue, green, yellow, etc.).

Chemical identification of microplastics

## ATR-FTIR spectroscopy

## 3.1.1) Prior to the analysis clean the detection system with alcohol and a lint free cloth.

## 3.1.2) Record a background spectrum. Place the sample on the sample holder and collect the spectra. Identify the obtained ATR- FTIR spectra using an automated comparison of the obtained spectrum with spectra in a database.

## Micro ATR-FTIR spectroscopy

## 3.2.1) Prior to the analysis clean the detection system with alcohol and a lint free cloth.

## 3.2.2) Place the sample on a glass filter. Note: Other filters can be used but their polymer nature can interfere with the characterization.

## 3.2.3) Place the filter with the sample on the automatic scanning table and use the joystick to locate the sample.

## 3.2.4) Record an optical image and mark an area (e.g. 20 by 20µm) where the sample will be characterized.

## 3.2.5) Record a background spectrum.

## 3.2.6) Place the sample on the sample holder and collect the spectra at the predefined location.

## 3.2.7) Identify the obtained micro ATR-FTIR spectra using an automated comparison of the obtained spectrum with spectra in a database.

**REPRESENTATIVE RESULTS:**

The first result of the described protocol are microplastic particles categorized into six categories according to their visual features (Table 1). The first category, and usually the most abundant one, are fragments (Figure 1). They are rigid, thick, with sharp crooked edges and an irregular shape. They can be in a variety of different colors. The second category are films (Figure 2). They also appear in irregular shapes, but in comparison with fragments, they are thin and flexible and usually transparent. The third category are pellets (Figure 3), usually originating from the plastics industry. They are irregular, round shapes, and normally bigger in size, around 5 mm in diameter. They are usually flat on one side and can be of various colors. The fourth category are granules (Figure 4). In comparison with pellets, they have a regular round shape and usually a smaller size, around 1 mm in diameter. They appear in natural colors (white, beige, brown). The fifth category are filaments (Figure 5). They are, next to fragments, the most abundant type of microplastic particles. They can be short or long, with different thicknesses and colors. The last category are foams (Figure 6). They most often come from large particles of styrofoam. They are a soft, irregular shape and white to yellow in color.

The main result of microplastics sampling and sample analysis is the number of microplastic particles per sample. These data can be further normalized per km2. The formula used for normalization is:

Microplastic particles per sample / sampling area, where sampling area is calculated by multiplying sampling distance by the width of the opening of the manta net (Tables 2,3; Figure 7). In addition, particles can be analyzed with image analysis software. The results include maximum length and area of each particle (Table 4). Figure 8a show particles before image analysis and Figure 8b is after image analysis, where each particle is measured and numbered. Lastly, a chemical analysis of the total or highest possible number of particles per sample is recommended. Using Fourier transform infrared spectroscopy a spectrum of the selected particle is acquired, as shown on Figure 9. This spectrum is then compared with the spectra from the software library (Figure 10). The final result will show if a given particle is plastic or not and indicate the type of plastic from the chemical structure.

**TABLE AND FIGURE LEGENDS:**

**Table 1: Categories of Microplastic particles**

[Place Table 1 here]

**Figure 1: Example of particles from category: Fragments**

[Place Figure 1 here]

**Figure 2: Example of particles from category: Films**

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**Figure 3: Example of particles from category: Pellets**

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**Figure 4: Example of particles from category: Granules**

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**Figure 5: Example of particles from category: Filaments**

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**Figure 6: Example of particles from category: Foams**

[Place Figure 6 here]

**Table 2: Example of data from survey, used for calculation of microplastic particles per km2**

[Place Table 2 here]

**Table 3: Example of results from survey, where the categorized data into 6 groups are counted and normalized per km2 (No - number of particles)**

[Place Table 3 here]

**Figure 7: Example of representative results after visual categorization of particles (No = number of particles)**

[Place Figure 7 here]

**Table 4: Example of image analysis results where area [mm2] and maximum length [mm] of each particle are measured**

[Place Table 4 here]

**Figure 8: Example of image acquired a) before and b) after image analysis of particles with image analysis software**

[Place Figure 8 here]

**Figure 9: Example of a spectra measured on a selected particle with marked peaks and their wavelengths [cm-1]**

[Place Figure 9 here]

**Figure 10: Example of comparison of acquired spectra from selected particle to best match from the ATR-FTIR spectra library**

[Place Figure 10 here]

**DISCUSSION:**

Microplastics sampling on the sea surface by manta net is a widely used method for the sampling of microplastics on the sea surface, but to date there has been no unified methodology. A large volume of water can be filtered through the manta net, thus the possibility of trapping a relevant number of microplastics is high and the results are perceived to be reliable. Comparability of results among different samples is assured by normalization. In our case, the concentrations were related to the sampled area by multiplying trawl distance by the horizontal width of the net opening. Another option is to use a flow meter, fixed at the net opening. The use of a flow meter is possible since the manta net with its lateral wings is very stable on the sea surface and therefore hopping on the waves is minimal. A flow meter records the volume of filtered water and thus enables the normalization of results per volume of sampled water16.

The most frequently used manta nets have around 300 µm mesh size and are 3 – 4.5 m long. These dimensions were optimized to avoid clogging of the net and to allow the sampling a volume of water as large as possible. Trawling speed is recommended to be between 2 – 3 knots, but it is dependent on wave height, wind speed and sea currents. It is very important that the manta net is under supervision the whole time during sampling and if it starts hopping, the trawling speed must be reduced. The trawling time is recommended to be around 30 min, but depends on seston concentrations. It can happen that seston sometimes clogs the manta net. In this case the trawling has to be stopped immediately, otherwise the microplastic particles can be lost and the net can get damaged. Manta net is the most often fixed from the side of the vessel. This is also the most suitable option, while the manta net is surely out of the wake zone. In some surveys manta net was fixed from the stern of the vessel17, 18, but in that case you have to be sure that the net is out of the wake zone. The distance, on which the trawl is set for sampling, should be determined individually, since the zone of turbulences caused by the vessel varies from the size of the vessel and from the speed of the boat19, 20.

Separation of microplastic particles from the sea surface samples is most often done just by visual identification21. Particles bigger than 1 mm can be identified easily by the naked eye, while particles smaller than 1 mm require the use of a stereomicroscope. To reduce the possibility of confusing the non-plastic particles with plastic ones, using the polarization light on stereomicroscopes is recommended. The possibility of misidentification of plastic particles gets higher with smaller particles. Thus particles >0.5 mm can only be identified visually21, by the use of stereomicroscope. For particles smaller than 0.5 mm an additional, more accurate method is required e.g. micro ATR-FTIR spectroscopy21.

During the process of microplastics separation from the sample the possibility of sample contamination with the airborne filaments is very high. For this reason, control Petri dishes left open on the working table are strongly recommended for the identification of potential contaminant airborne particles. Namely, the quality of the data strongly depends on: 1) the precision of the person working with the sample, 2) the quality and magnification of the stereomicroscope and 3) the quantity of organic matter in the sample16. After visual identification it is strongly recommended to analyze the sorted particles with one of the available techniques for chemical identification of the material8.

Several methods exist for polymer identification, among which the FTIR spectroscopy and Raman spectroscopy are the most frequently used22. FTIR and Raman spectroscopy are complementary techniques and their accuracy is similar. In our protocol, the FTIR and micro FTIR spectroscopy with “attenuated total reflectance” (ATR) are presented. They are simple to use and they enable fast and accurate results. Plastic polymers possess highly specific infrared (IR) spectra with distinct band patterns, thus making IR spectroscopy an optimal technique for the identification of microplastics21. The energy of IR radiation excites a specific molecular vibration when interacting with a sample, which enables the measurement of characteristic IR spectra22. FTIR spectroscopy can also provide additional information on particles, such as intensity of oxidation23 and level of degradation24. While ATR-FTIR is suitable for chemical identification of larger particles (>0.5 mm), micro ATR-FTIR spectroscopy can provide information on the chemical structure of particles <0.5 mm, as it combines the function of a microscope and an infrared spectrometer.

Before using FTIR and micro FTIR spectroscopy, microplastic particles have to be previously dried, since water strongly absorbs IR radiation22, and purified, in case they are covered with biofilms and/or other organic and inorganic adherents, which can influence the IR spectra. The most non-invasive way to purify samples is by stirring and rinsing with fresh water25. If this is not enough, then the use of 30 % hydrogen peroxide is recommended. All other methods can have negative effects on the microplastic particles (e.g. ultrasonic cleaning can further break particles, strong acidic or alkaline solutions can damage several plastic polymers, etc.) and therefore their use is not recommended. More promising is the use of a sequential enzymatic digestion as a plastic friendly purification step. Purification using different technical enzymes (e.g. lipase, amylase, proteinase, chitinase, cellulase, proteinase-K) has been successfully applied to reducing a biological matrix of plankton and thus proved to be a valuable technique to minimize matrix artifacts during FTIR spectroscopy measurements22.

Separation of microplastics by visual identification and chemical identification of selected particles are both extremely time-consuming processes. This work has to be done by an accurate and patient person who has experience with stereomicroscopes, not only in recognizing the plastic particles, but also in recognizing biological matter. Even an experienced person cannot discriminate all potential microplastic particles unambiguously from chitin or diatom fragments22. Therefore, the error rate of visual sorting ranges from 20 %26 to 70 %21 and increases with decreasing particle size.

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**DISCLOSURES:**

The authors have nothing to disclose.

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