Bases para la planificación sostenible de áreas marinas en la Macaronesia
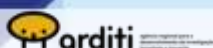
SAMPLING AND PROCESSING MICROPLASTICS FROM SURFACE WATERS

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PROYECTO COFINANCIADO POR LA UNIÓN EUROPEA

MAC 2014-2020 Cooperación Territorial

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To cite this report:

Herrera, A.; Martínez, I.; Gómez, M.; Rapp J.; Raymond E.; Álvarez, S.;Gestoso, I.; Canning-Clode, J. 2019. Sampling and processing microplastics from surface waters. Universidad de Las Palmas de Gran Canaria, Agência regional para o desenvolvimento da investigação, tecnología e inovação. Report prepared as part of PLASMAR Project (co-financied by ERDF as part of POMAC 2014-2020). 22 pp.

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1 Sampling process

1.1 Sampling

1.1.1 Equipment

1) Manta net (Fig. 1):

   - Stainless Steel structure with a front opening of 60x40cm and rear opening of 60x25cm.
   - On the sides, two ailerons also made of stainless steel (although there are modifications such as the use of buoys (Fig. 2)) of 60x40 cm.
   - 200 µm pore-mesh is placed in the rear opening (this measure could vary depending on the sample to be collected) with a collector at the end of it as shown in Figure 1. The collector will be covered with foam or other material that allows its flotation in horizontal position during the trawl of the net.
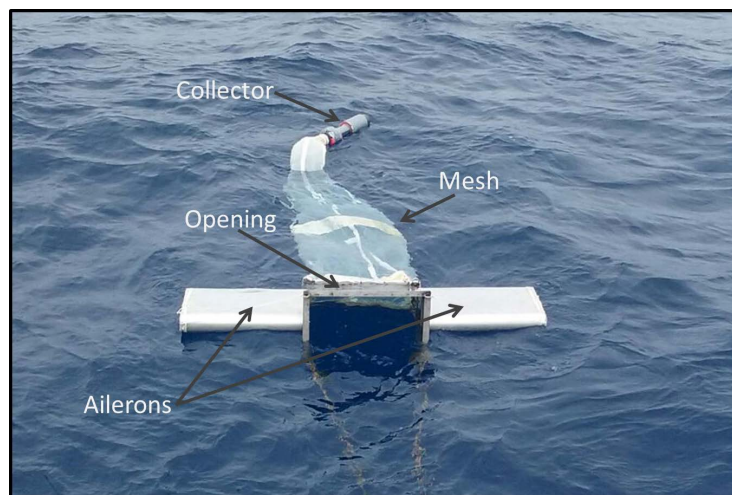
Figure 1. Manta net picture and its parts
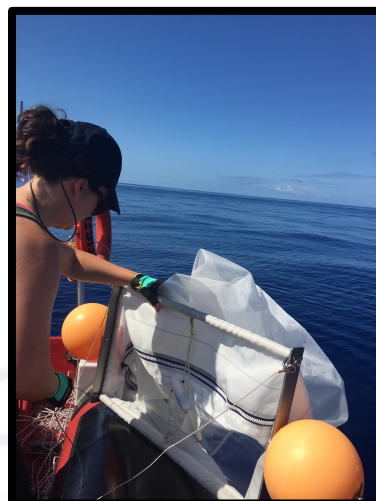
Figure 2. Picture of a manta net made with buoys instead of ailerons, and rear opening of 70X40cm.

2) Materials:

- Washing bottle with sea water
- 250mL bottles to collect the sample
- Funnel
- 50 µm mesh
- Formaldehyde 40% (if zooplankton collected will be taking in account)
- Buckets
- Sampling template (notes of transects location, weather, unexpected events, …)

1.1.2 Sampling procedure

1) Establishing and defining sampling area: geo-referencing (latitude and longitude) transects to be carried out, to create a map of the study area. One mile transects will be defined (Fig. 3).

Figure 3. Geo-location example. Each blue line corresponds to a one mile transect.

2) At the starting point of each transect, the net is launched and dragged at 3 knots of speed to complete the designated mile. Figure 4 shows the moment that net is picked up once transect finished.

Figure 4. Time of net collection in the vessel.

1.2   Sample collection

1) Sample collection system is prepared during net trawl: funnel; net pieces of 50 µm pore mesh to be placed in the funnel and filter the collector sample; properly labeled bottles; washing bottle with seawater; formaldehyde (Fig. 5).

Figure 5. Sample collection system

2) At the end of transect, take the net out of the water carefully, rinse well the mesh with seawater to complete the sample collection, and remove the collector. Pour the content of the collector with care in the funnel with the mesh (Fig. 6). Help yourself with the washing bottle with seawater, and pool the sample in the center of the mesh to facilitate the transfer to the bottles.

NOTE: If washing the net is not an option because there is no system with enough pressure to perform the washing in the vessel, and the collected zooplankton will not be taken into account, a detachable zippered net could be made (Fig. 2). This way, once the contents of the collector have been collected, the mesh can be removed, stored and the process continued on land.
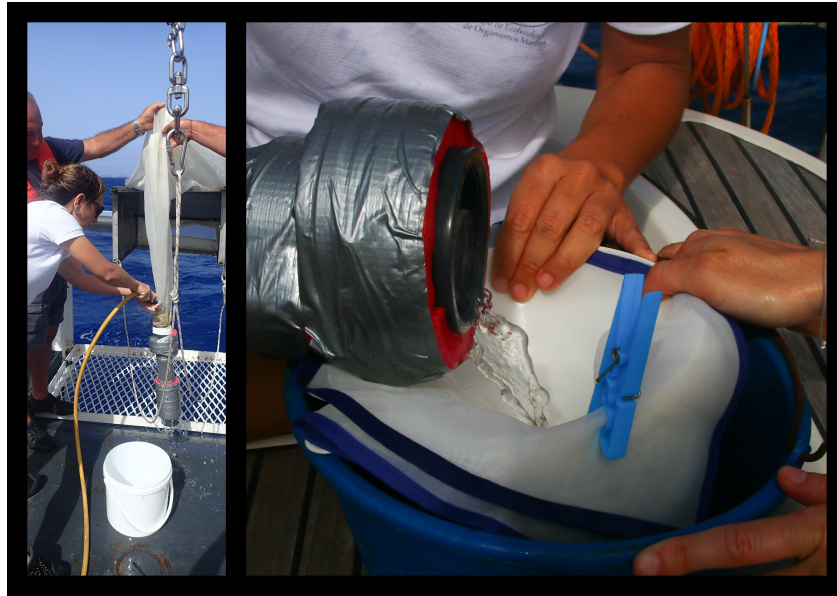
Figure 6. Sample collection. Left panel: mesh rinse before collector removing; Right panel: samples collection in the funnel with the 50µm pore mesh.

3) Once the sample is collected, transfer it from the 50µm mesh to its corresponding labeled bottle, helping yourself with the seawater washing bottle. Fill up until 225ml and complete it with 25ml of formaldehyde (40%). In this way, the samples will be preserving in 4% formaldehyde.

2 Sample processing

2.1   Security

Before treating the samples, make sure that the working area is exclusively for this purpose and is as sterile as possible to avoid external contamination. Synthetic fibers are found almost everywhere (Lusher et al., 2017).

- Sterilize and clean properly all working utensils
- Clean with alcohol the sample treatment zone
- Use cotton lab-coat

2.2   Sample processing

2.2.1   Sieving

a) Prepare three new bottles for each sample to sieve; obtaining three sub-samples (Fig. 7.1).

b) To sieve, put the sieving-column on a beaker-fitted funnel (Fig. 7.2; excess of water is collected in that beaker). The column consists of three sieves:
   1. *Upper sieve:* with a 1000 µm pore mesh (size range ***>1000 µm***)
   2. *Intermediate sieve:* with a 500 µm pore mesh (size range ***500-1000 µm***)
   3. *Lower sieve:* with a 200 µm pore mesh (size range ***200-500 µm***)

   Thus, three different size ranges for each collected sample are obtained, and those ranges are applicable both organisms and microplastics.

c) Once the sieving-column is well positioned on the funnel, pour the sample and rinse with bi-distilled water as many times as necessary to transfer the entire sample. When finished, spray the upper sieve with seawater using a pressure sprayer (Fig. 7.3) to achieve that all 1000µm lower organisms/MP pass through the sieves. In this way, the upper size range overestimation and lower size range underestimation is avoided. This last step will be repeated with the 500 µm sieve.

d) Separate the sieves and store each subsample in its corresponding bottle using a funnel placed in the bottle's opening. Here, pour the content of the sieve, with the help of the bi-distilled water washing bottle to transfer the sample (Fig. 7.5).
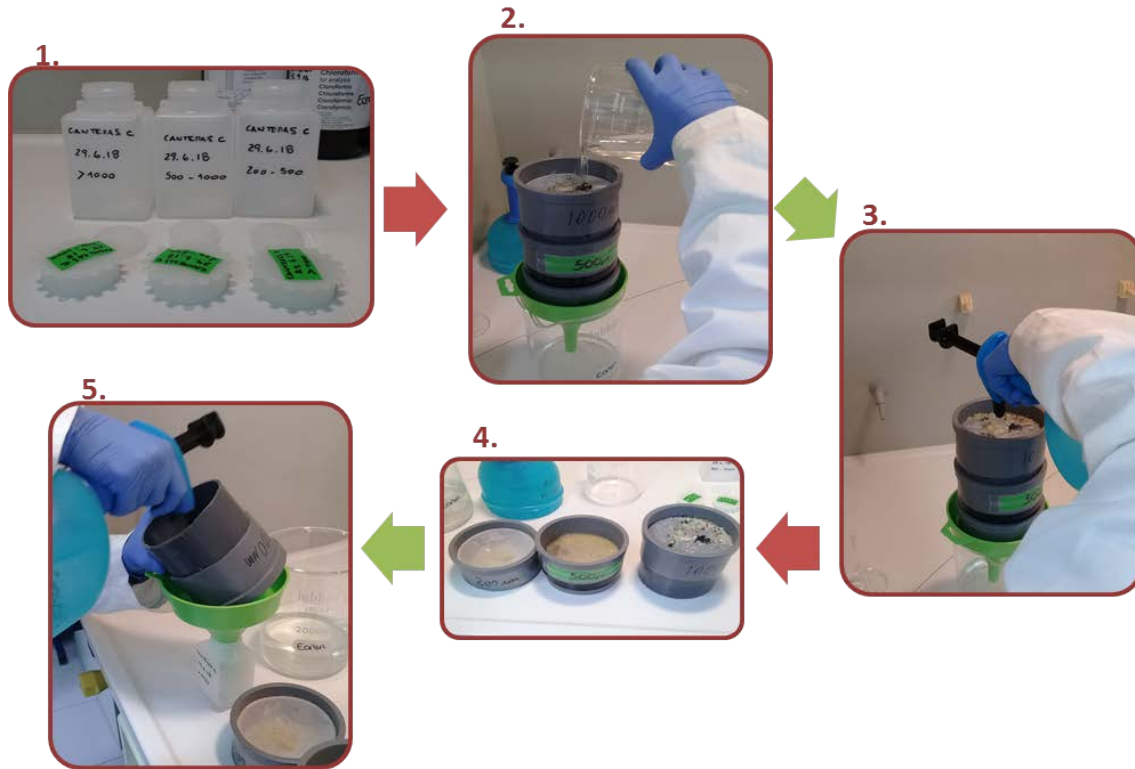
Figure 7. Sieving and size range separation process.

2.2.2 Sub-sample filtering and counting

a) Prepare small circle filters from a 50μm mesh net, with a diameter of 90mm like a Petri dish.

b) Filter each sample. Place the filter in a small funnel (Fig. 8) and pour the bottle content. After this, remove the filter with care, put it in the Petri dish and, with a herringbone needle, split the content for better counting in the stereomicroscope. Store the remaining liquid to reuse it to store the sample.
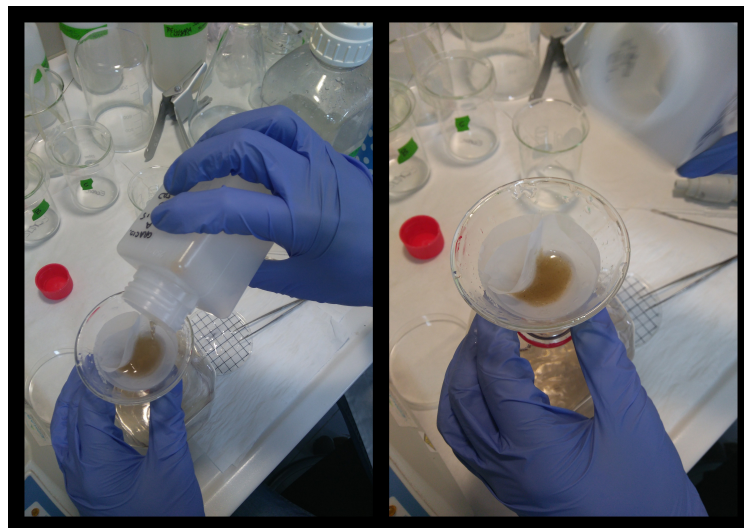
Figure 8. Filtering process of each sub-sample

c) Place the Petri dish in the stereomicroscope and proceed with both microplastic counting and classification (Fig.9). Near the stereomicroscope it is necessary to place an empty Petri dish as the blank, to test the outside fiber contamination.
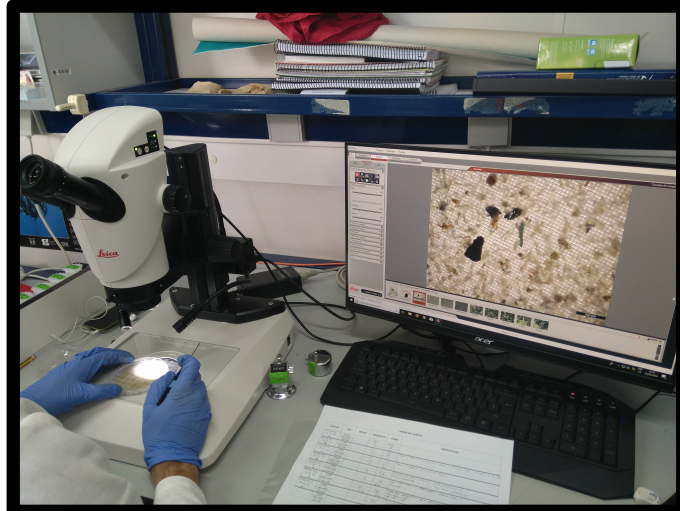
Figure 9. Microplastics counting with a stereomicroscope

Founded microplastics will be classified according to typology and color as defined in Table 1.

NOTE: If zooplankton concentration will not be taken into account, it is possible to do a digestion process of the organic material before 2.2.2 step to make the counting easier.

Table 1. Classification keys for seawater-surface microplastics. "Lines": presence of both fibers or cord lines from fishery nets; "Films": refers to bags fragments; "Fiber contamination": fibers that could appear in the blank.

SAMPLE	TYPE	Red	Brown	Orange	Yellow	Green	Blue	Purple	Pink	Silvered	Gray	Black	Semitransparent	Transparent	White	TOTAL	NOTES
	Fibers															0	
	Fragments															0	
	Lines															0	
	Films															0	
	Fiber contamination															0	
	Fibers															0	
	Fragments															0	
	Lines															0	
	Films															0	
	Fiber contamination															0	
	Fibers															0	
	Fragments															0	
	Lines															0	
	Films															0	
	Fiber contamination															0	
	Fibers															0	
	Fragments															0	
	Lines															0	
	Films															0	
	Fiber contamination															0	

d) After counting and classifying, store the sample in its corresponding bottle.

2.2.3 Zooplankton counting and identification

2.2.3.1 Equipment and software

1) Computer:
   - Processor of 3GHz or higher
   - Minimum of 1-2GB RAM memory.
   - Screen with a minimum size of 19"
   - Fast graphic card, able to show 1600x1200 pixels with a color depth of 24732bits
   - Hard disk with, at least, 200GB of memory
   - It is recommended to have a security system, i.e. with external hard disks of 200 or 300GB to store processed images
   - O.S. Windows 7 or higher

2) Scanner (with enough resolution to allow to capture the highest quality images as possible):
   - Flat scanner
   - Scan resolution: 6400 ppp (horizontal x vertical)
   - Optical resolution: Primary 6400ppp x Secondary 9600ppp
   - Optical density: 4Dmax
   - Scan interval: 216mm x 297mm (horizontal x vertical)
   - Color depth: in: 48 Bits Color, out: 48 Bits Color
   - Category: High resolution

3) Software Vuescan Pro (https://www.hamrick.com/)

4) Software Zooprocess (https://sites.google.com/view/piqv/)

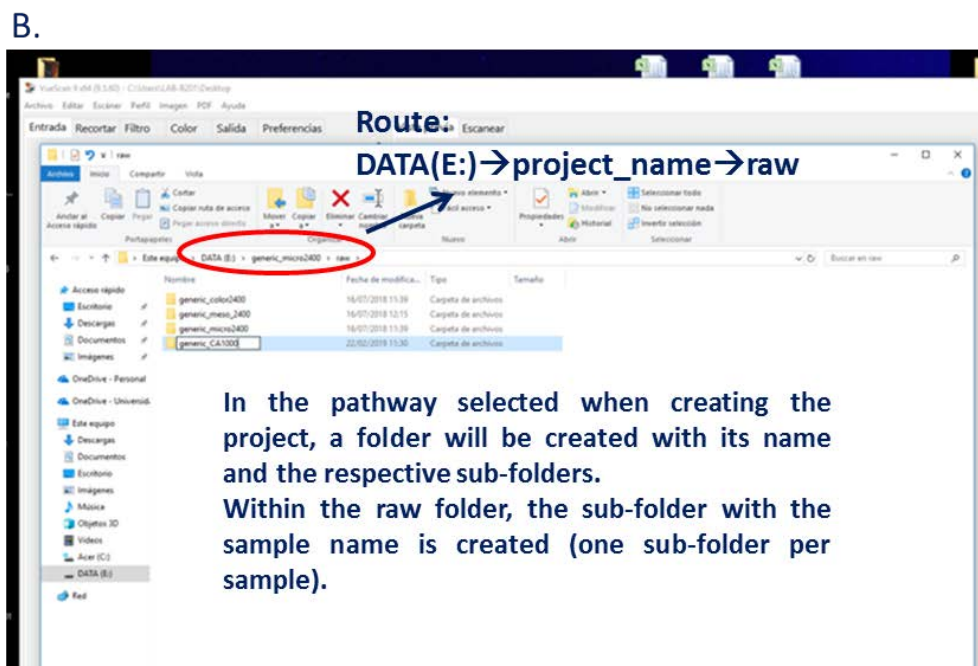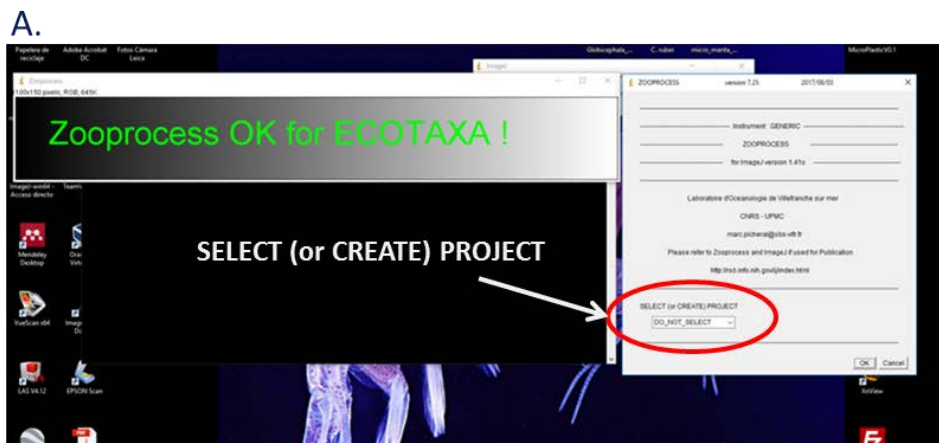#### 2.2.3.2 Sample processing

a) Prepare small circle filters from a 50μm mesh net, with a diameter of 90mm like a Petri dish.

b) Prior starting the sample processing, the project where images will be processed must be created, and the required scan parameters will be established:

   1. Execute Zooprocess program and create a new project (Fig. 10A). This step generates a folder with the project name in the choosing pathway (Fig. 10B), and the images will be stored in it. Close the program once the project has been created.
   NOTE: By default, several sub-folders also generate within the created folder when creating the new Project (config, docs, ecotaxa, meta, PID_process, raw, results, work).

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2. Define the name of each sample file: **ZAnº**, where **Z** is the sampling zone, **A** is the replicate, and **nº** indicates the size range.
3. In the new project *raw* folder, create a sub-folder with the name of the sample that will be scan (it will create one sub-folder by sample) (Fig. 10B).
4. Open the Vuescan application (Fig. 11), and specify the scan parameters (transparency mode, 2400ppp, .jpg format). Copy and paste the pathway of the folder created before as the out pathway (where the scanned image is to be stored after scanning, i.e. E://projectname/raw/ZAnº).
5. Leave everything ready, and proceed to prepare the sample.

A.

B.

Figure 10. (A) New Zooprocess project for samples processing; (B) Example of sub-folder creation with the sample's name
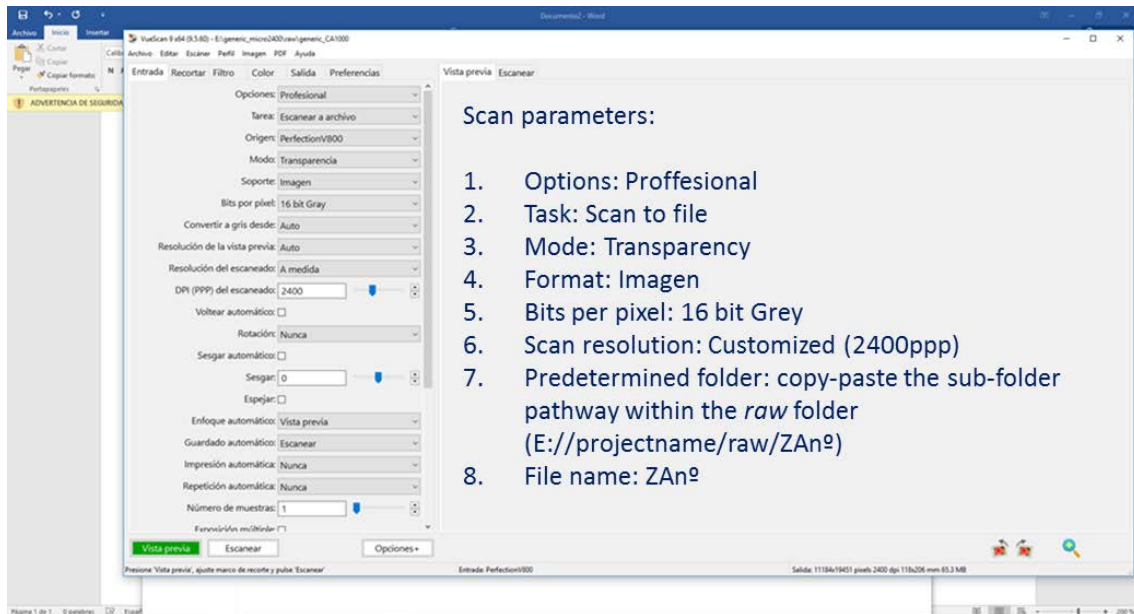
Figure 11. Vuescan parameters established before scanning.

c) Filtering each sample. Place the filters in a small funnel, and pour the bottle content (Fig. 8). Concentrate the sample in the center of the filter using the washing bottle. Transfer it to a beaker containing 100ml of bi-distilled water.
NOTE: If the samples are highly concentrated, 200ml of bi-distilled water can be added.

d) Homogenate the sample well, and take a 10ml (10%) or 20ml (20%) aliquot .The 20% aliquot is in the case of the sample is too diluted.

e) Spread out the aliquot over the methacrylate tray (Fig. 12), and place it in the scanner.
NOTE: Make sure that the tray is properly positioned so that there is no error when scanning (e.g. the scanner cover is incorrectly closed, etc…).
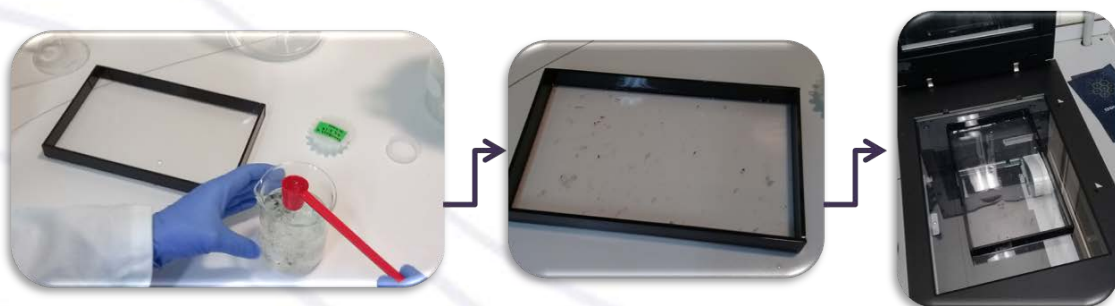

Figure 12. Sample scanning process

f) Scan the sample, and when finished, return the sample to the corresponding bottle. In that bottle, the samples must remain with a proportion of 225ml of bi-distilled water and 25ml of formaldehyde, to store it at 4% formaldehyde concentration.

NOTE: Scanned images must be in .TIFF format to avoid errors with the Zooprocess software.

g) Check that the corresponding file is created, and also created in the correct folder.

2.2.3.3 Image processing

Once all images are acquired:

a) Execute the Zooprocess software, and select the corresponding project (Fig. 10A).

b) Change to advanced mode, and fill in the sample data (Fig. 13). When 'Metadata' is filled, keep the 'Sampling date', 'Lat' and 'Long' parameters with the exact format established by the software to avoid mistakes with the image processing.
In the *"Concentrated water vol."* box, it is desirable to write the volume in which the sample was concentrated (100 or 200 mL), and in "*Comment"* add aliquot volume placed in the methacrylate tray (10 or 20mL). Then, once all interesting data are filled in, press Ok.

c) Select "PID and VIGNETTES" in SELECT PROCESS window. Check that all configuration file data are right (e.g. if works at 2400ppp, the resolution must be pixel= 10.6). If everything is correct, the program will process the sample, and separate each item that it finds in the vignettes (Fig. 14). These vignettes will be stored in the ***work*** project sub-folder, within a folder with the sample's name.
NOTE: In case that configuration data does not coincide, cancel, and back to SELECT PROCESS. Go into "Edit Configuration File", and correct the wrong data.
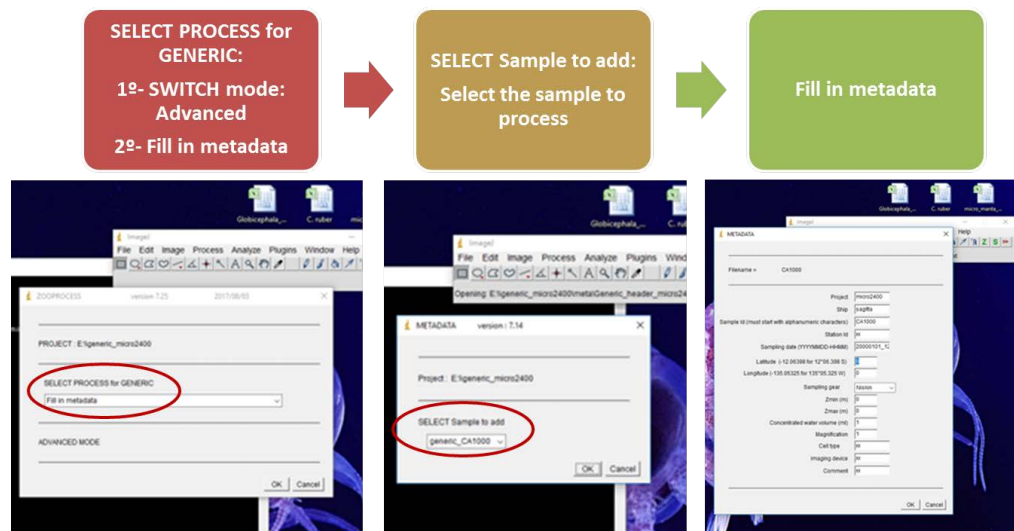*ESD: minimum and maximum organisms detected size.*

Figure 13. First steps to start with the image processing. Enter in Advanced mode and fill in metadata.
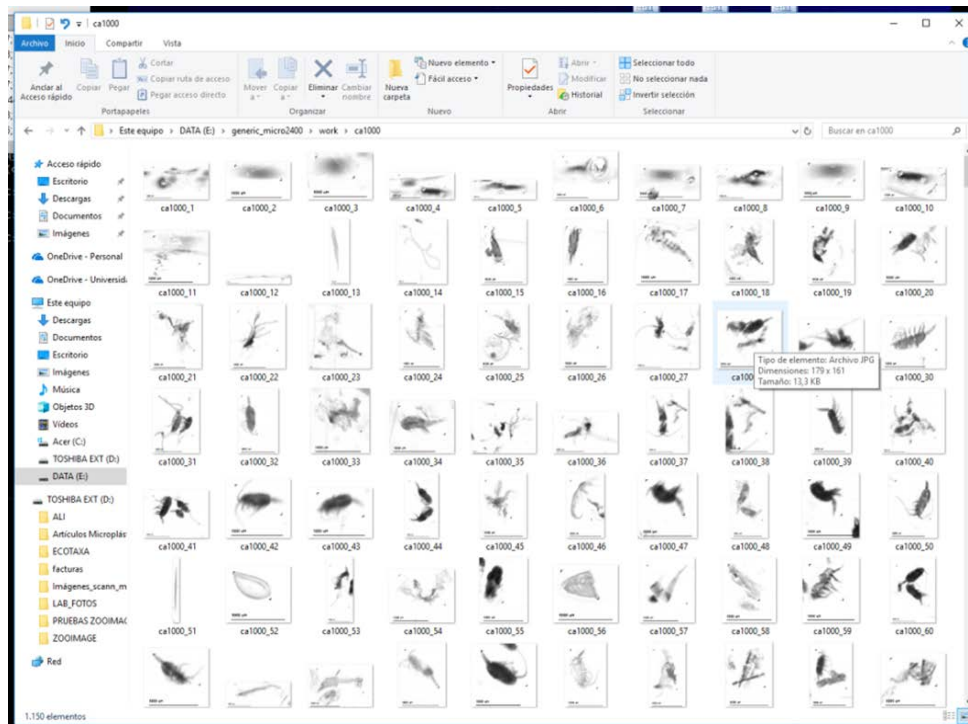
Figure 14. A Vignettes example once samples have been processed.

d) After vignettes have been created, the organisms are identified and counted.

A tool for this objective is the web application ECOTAXA (https://ecotaxa.obs-vlfr.fr/). The first steps to follow to work with this tool would be:

1) Execute Zooprocess program, in the SELECT PROCESS initial window select the "CREATE TABLES FOR ECOTAXA" option, and in the next step, keep the default option (SELECT OPTION: ALL unpredicted/validated vignettes from "work" subfolders).

2) Files created will be stored in the *work* sub-folder.

3) Enter in ECOTAXA web, registered to create a new project, and manage the images as appropriate. It will be possible to export final data in .tsv format (Excel compatible format), including all data of each organism: area, perimeter, width, length, roundness, etc. (Fig 15).

4) In the web page https://sites.google.com/view/piqv/ecotaxa are the protocols to consult how to import/export data and images.

Figure 15. Example of data and vignettes obtained with ECOTAXA processing method. a) vignettes; b) summary table; c) Excel table example

References

Lusher A.L., Welden N.A., Sobral P., Cole M., 2017. Sampling, isolating and identifying microplastics by fish and invertebrates. Analytical Methods, 9: 1346-1360