Journal of Visualized Experiments

Protocol for microplastics sampling on the sea surface and sample analysis

--Manuscript Draft--

Manuscript Number:	JoVE55161R1
Full Title:	Protocol for microplastics sampling on the sea surface and sample analysis
Article Type:	Methods Article - Author Produced Video
Keywords:	Microplastics; protocol; sea surface; microplastics sampling; manta net; microplastics analysis; FTIR spectroscopy; micro FTIR spectroscopy.
Manuscript Classifications:	7.16.500: Ecological and Environmental Phenomena; 7.16.500.275: Environment; 7.16.500.275.810: Seawater; 8.1: Natural Science Disciplines; 8.1.158: Biological Science Disciplines; 8.1.158.273: Biology; 8.1.158.273.248: Ecology; 8.1.277: Earth Sciences; 8.1.277.249: Ecology
Corresponding Author:	Manca Kovač Viršek Institute for water of the Republic of Slovenia Ljubljana, Ljubljana SLOVENIA
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	manca.virsek@izvrs.si
Corresponding Author's Institution:	Institute for water of the Republic of Slovenia
Corresponding Author's Secondary Institution:	
First Author:	Manca Kovač Viršek
First Author Secondary Information:	
Other Authors:	Andreja Palatinus
	Špela Koren
	Monika Peterlin
	Petra Horvat
	Andrej Kržan
Order of Authors Secondary Information:	
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.
Author Comments:	This video production was financed by IPA Adriatic Programe within the DeFishGear project. Because this publication fee can be financed only from the DeFishGear project which will end up on 30th of September 2016, we kindly ask you to publish the video till this date.
Additional Information:	

Question	Response
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	09-30-2016

Manca Kovač Viršek, PhD
Sector for marine waters
Institute for water of the Republic of Slovenia
Dunajska cesta 156, SI-1000 Ljubljana
Slovenia

Ljubljana, 24th of June 2016

Mrs. Alison Hamlin, Associate Editor – Environment
MyJoVE Corp.
One Alewife Center, Suite 200
Cambridge, MA 02140

Dear Mrs. Hamlin,

We wish to submit a manuscript entitled "Protocol for microplastics sampling on the sea surface and sample analysis" for consideration by the JoVE.

In this paper, we report on methodology for microplastics sampling on the sea surface by manta net, sample preparation, separation and chemical identification of microplastic particles. This video with manuscript should be of interest to readers that deal with microplastic monitoring all over the world.

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

This video production was financed by IPA Adriatic Programe within the DeFishGear project. Because this publication fee can be financed only from the DeFishGear project which will end up on 30th of September 2016, we kindly ask you to publish the video till this date.

All other information, about author contribution, JoVe editors who have assisted us and list of six peer reviewers are described below.

Please address all correspondence concerning this manuscript to me at manca.virsek@izvrs.si.

Thank you for your consideration of this manuscript.

Sincerely,

Manca Kovač Viršek

1) Author contributions
Kovač Viršek, Manca – coordinator, protocol development, video filming, manuscript writing

Palatinus, Andreja – protocol development, video filming

Koren, Špela – manuscript writing and editing, photo contribution

Peterlin, Monika – manuscript editing

Horvat, Petra - protocol development, video filming, manuscript writing

Kržan, Andrej - protocol development, proofreading

2) The names of any JoVE editors who have assisted you in the submission process:

Alison Hamlin; Associate Editor – Environment; alison.hamlin@jove.com

Initial contact person:
Mark Ingram; Director of Sales | EMEA; mark.ingram@jove.com

3) List of 6 peer reviewers' names with their institutional affiliation

Amandine Collignon
University of Liège, Laboratory of Oceanology – MARE Research Center, Allée de la Chimie 17, B6C, Institut de Chimie, 4000 Liege (Sart-Tilman), Belgium. Tel.: +32 (0) 496 43 05 85.
amandine.collignon@ulg.ac.be

François Galgani
Institut français de recherche pour l'exploitation de la mer (Ifremer), 83507 La Seyne-sur-Mer Cedex, Corse, France
Francois.Galgani@ifremer.fr

Martin G. J. Löder
Animal Ecology I, University of Bayreuth
Biologische Anstalt Helgoland, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Marine Station, Germany
Martin.Loeder@uni-bayreuth.de

Martin Thiel
Facultad Ciencias del Mar, Universidad Católica del Norte, Larrondo 1281, Coquimbo, Chile
thiel@ucn.cl.

Da Ros Luisa
Istituto di Scienze Marine, ISMAR-CNR, Castello 1364/a, 30122 Venezia, Italy
luisa.daros@ismar.cnr.it

Gaby Gorsky
Observatoire océanologique de Villefranche-sur-mer
181 Chemin du Lazaret, 06230 Villefranche-sur-Mer, France
gorsky@obs-vlfr.fr

Prof. Maria Cristina Fossi
Department of Physical science, earth and environment
Univeritá di Siena, Via Mattioli 4, Siena 1240, Italy
fossi@unisi.it

Julia Reisser
Ocean clean up / The University of Western Australia / CSIRO - Commonwealth Scientific and Industrial Research Organisation
CSIRO, Private bag 10, Clayton South Vic 3169, Australia
Julia.Reisser@csiro.au

TITLE:
Protocol for microplastics sampling on the sea surface and sample analysis

AUTHORS:
Kovač Viršek, Manca
Sector for Marine Waters
Institute for water of the Republic of Slovenia
Ljubljana, Slovenia
manca.virsek@izvrs.si
+386 (0)1 47 75 301

Palatinus, Andreja
Sector for Marine Waters
Institute for water of the Republic of Slovenia
Ljubljana, Slovenia
andreja.palatinus@izvrs.si

Koren, Špela
Sector for Marine Waters
Institute for water of the Republic of Slovenia
Ljubljana, Slovenia
spela.koren@izvrs.si

Peterlin, Monika
Sector for Marine Waters
Institute for water of the Republic of Slovenia
Ljubljana, Slovenia
monika.peterlin@izvrs.si

Horvat, Petra
Laboratory for Polymer Chemistry and Technology
National Institute for Chemistry Slovenia
Ljubljana, Slovenia
petra.horvat@ki.si

Kržan, Andrej
Laboratory for Polymer Chemistry and Technology
National Institute for Chemistry Slovenia
Ljubljana, Slovenia
andrej.krzan@ki.si

CORRESPONDING AUTHOR:
Manca Kovač Viršek

KEYWORDS:
microplastics, protocol, sea surface, microplastics sampling, manta net, microplastics analysis, FTIR spectroscopy, micro FTIR spectroscopy

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 environment[1]. 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 sea[2]. The majority of microplastic pollution studies were carried out in marine and fresh water ecosystems and mainly addressed sea surface pollution[3].

The term microplastic refers to plastic particles smaller than 5 mm in size[4]. 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 origin[5]. 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 organisms[6]. In 2015, four types of microplastic sources were defined: larger plastic litter, cleaning products, medicines and textiles[6]. The main source (80 %) of larger plastic litter is assumed to be land based[7]. Microplastic from cosmetic products, medicines and textile enters water ecosystems through sewage and storm waters[6]. Microplastic particles most frequently found in water ecosystems are fragments from larger plastic litter and textile fibers[8].

Microplastics have several negative effects on the environment. Their small size allows them to enter the food web through ingestion by marine organisms[9, 10]. Ingested particles can cause physical damage or block the digestive system of animals[11]. 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 microplastics[12]. In the environment or digestive systems of animals who ingest them, POPs and other plastic additives can be leached from microplastic particles[13].

Previous studies reported the ubiquitous presence of microplastics in the marine environment[3], 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 advised[14]. Accordingly, the EU Technical Subgroup on Marine Litter (TSG-ML) prepared recommendations for monitoring of microplastics in the European seas[15]. 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-ML[15] 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 waters[16]. 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:

1 Sampling of microplastics on the sea surface

1.1) Deploy the manta net from the side of the vessel using a spinnaker boom or »A-frame« using lines and karabiners.

1.2) 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.

1.3) Write down the initial GPS coordinates and initial time in the data sheet.

1.4) Start to move in one straight direction with a speed of approx. 2 - 3 knots for 30 min and begin the time measurement.

1.5) 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.

1.6) 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.

1.7) Safely remove the cod end and sieve the sample in the cod end through a 300 µm mesh size sieve or less.

1.8) 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.

1.9) Concentrate all material on the sieve in one part of the sieve.

1.10) With the use of a funnel, rinse the sieve into a glass jar or plastic bottle using 70 % ethanol.

1.11) 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 to general 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.

2 Separation of microplastics from the sea surface samples

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

2.2) 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 Items[16].

2.3) 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.

2.4) 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.

2.5) 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.

2.6) 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.

2.7) 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.).

3 Chemical identification of microplastics

3.1 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.

3.2 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 km^2. 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
[Place Figure 2 here]

Figure 3: Example of particles from category: Pellets
[Place Figure 3 here]

Figure 4: Example of particles from category: Granules
[Place Figure 4 here]

Figure 5: Example of particles from category: Filaments
[Place Figure 5 here]

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 km^2
[Place Table 2 here]

Table 3: Example of results from survey, where the categorized data into 6 groups are counted and normalized per km^2 (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 [mm^2] 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 water[16].

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 vessel[17, 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 boat[19, 20].

Separation of microplastic particles from the sea surface samples is most often done just by visual identification[21]. 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 visually[21], by the use of stereomicroscope. For particles smaller than 0.5 mm an additional, more accurate method is required e.g. micro ATR-FTIR spectroscopy[21].

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 sample[16]. After visual identification it is strongly recommended to analyze the sorted particles with one of the available techniques for chemical identification of the material[8].

Several methods exist for polymer identification, among which the FTIR spectroscopy and Raman spectroscopy are the most frequently used[22]. 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 microplastics[21]. The energy of IR radiation excites a specific molecular vibration when interacting with a sample, which enables the measurement of characteristic IR spectra[22]. FTIR spectroscopy can also provide additional information on particles, such as intensity of oxidation[23] and level of degradation[24]. 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 radiation[22], 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 water[25]. 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 measurements[22].

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 fragments[22]. Therefore, the error rate of visual sorting ranges from 20 %[26] to 70 %[21] and increases with decreasing particle size.

ACKNOWLEDGEMENTS:
The development of this protocol was founded by IPA Adriatic Cross-border Cooperation Programme 2007-2013, within the DeFishGear project (1°str/00010).

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1. Law, K. L., *et al*. Plastic accumulation in the North Atlantic subtropical gyre. *Science*. 329 (5996), 1185-1188, doi: 10.1126/science.1192321 (2010).
2. Thompson, R. C. Microplastics in the marine environment: Sources, consequences and solutions. In Marine anthropogenic litter. Springer International Publishing. 185-200, doi: 10.1007/978-3-319-16510-3_7 (2015).
3. Lusher, A. Microplastics in the marine environment: distribution, interactions and effects. In: Marine anthropogenic litter. Springer International Publishing. 245-307, doi: 10.1007/978-3-319-16510-3_10 (2015).

4. Arthur, C., Baker, J. & Bamford, H. Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, NOAA Technical Memorandum NOS-OR&R30 (2008).
5. Andrady, A.L. Microplastics in the marine environment. Marine pollution bulletin. 62(8), 1596-1605, doi: 10.1016/j.marpolbul.2011.05.030 (2011).
6. Browne, M.A. Sources and pathways of microplastics to habitats. In: Marine anthropogenic litter. Springer International Publishing. 229-244, doi: 10.1007/978-3-319-16510-3_9 (2015).
7. UNEP'S REGIONAL SEAS PROGRAMME. Marine litter: an analytical overview. UNEP, (2005).
8. van der Wal, M., et al. SFRA0025: Identification and Assessment of Riverine Input of (Marine) Litter, Final Report for the European Commission DG Environment under Framework Contract No ENV.D.2/FRA/2012/0025 (2015).
9. Setälä, O., Fleming-Lehtinen, V., Lehtiniemi, M. Ingestion and transfer of microplastics in the planktonic food web. Environmental pollution. 185: 77-83, doi: 10.1016/j.envpol.2013.10.013 (2014).
10. Farrell, P., Nelson, K. Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). Environmental Pollution. 177, 1-3, doi: 10.1016/j.envpol.2013.01.046 (2013).
11. Wright, S.L., Thompson, R.C., Galloway, T.S. The physical impacts of microplastics on marine organisms: a review. Environmental Pollution. 178: 483-492, doi: 10.1016/j.envpol.2013.02.031 (2013).
12. Bakir, A., Rowland, S.J., Thompson, R.C. Transport of persistent organic pollutants by microplastics in estuarine conditions. Estuarine, Coastal and Shelf Science. 140, 14-21, doi: 10.1016/j.ecss.2014.01.004 (2014).
13. Cole, M., Lindeque, P., Halsband, C., Galloway, T.S. Microplastics as contaminants in the marine environment: a review. Marine pollution bulletin. 62(12), 2588-2597, doi: 10.1016/j.marpolbul.2011.09.025 (2011).
14. Zarfl, C., et al. Microplastics in oceans. Marine Pollution Bulletin. 62, 1589-1591, doi: 10.1016/j.marpolbul.2011.02.040 (2011).
15. Hanke, G., et al. MSFD GES technical subgroup on marine litter. Guidance on monitoring of marine litter in European Seas. Luxembourg: Joint Research Centre-Institute for Environmente and Sustainability. Publications Office of the European Union. doi: 10.2788/99475 (2013).
16. Löder, M. G. J., Gerdts, G. Methodology used for the detection and indentification of microplastics – A critical appraisal. In: Marine anthropogenic litter. Springer International Publishing. 201 – 227, doi: 10.1007/978-3-319-16510-3_8 (2015).
17. Kang, J. H., Kwon, O. Y., Lee, K. W., Song, Y. K., Shim, W. J. Marine neustonic microplastics around the southeastern coast of Korea. Marine pollution bulletin. 96(1), 304-312, doi: 10.1016/j.marpolbul.2015.04.054 (2015).
18. Lusher, A. L., Tirelli, V., O'Connor, I., Officer, R. Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples. Scientific reports. 5, doi: 10.1038/srep14947 (2015).
19. Shu, J.-J. Transient Marangoni waves due to impulsive motion of a submerged body. International Applied Mechanics. 40 (6): 709–714, doi: 10.1023/B:INAM.0000041400.70961.1b (2004).

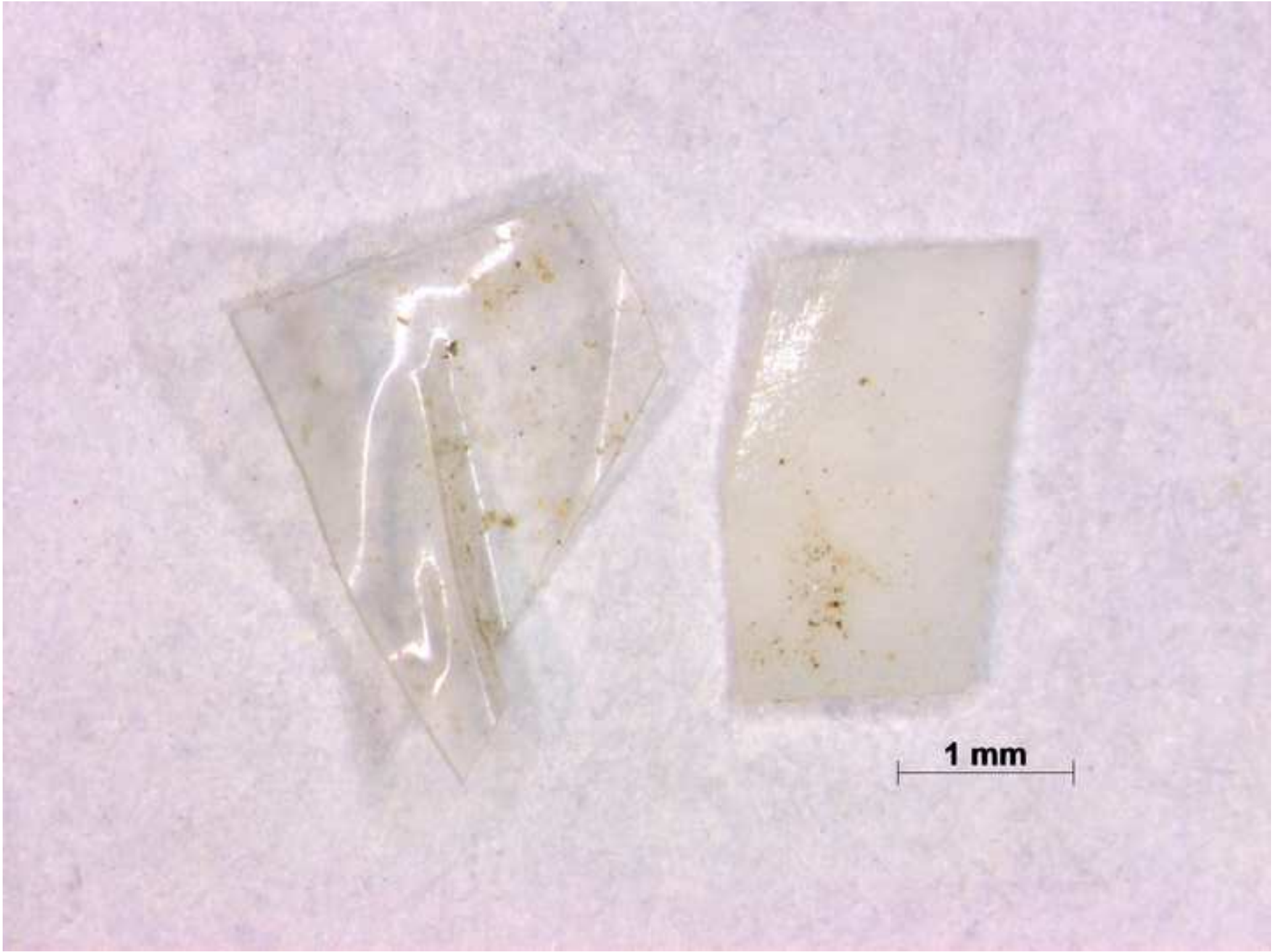
20. Rabaud, M., Moisy, F. Ship wakes: Kelvin or Mach angle?, Physical Review Letters. 110 (21): 214503, doi: 10.1103/PhysRevLett.110.214503 (2013).
21. Hidalgo-Ruz, V., Gutow, L., Thompson, R. C., Thiel, M. Microplastics in the marine environment: a review of the methods used for identification and quantification. Environmental science & technology. 46(6), 3060-3075, doi: 10.1021/es2031505 (2012).
22. Löder, M. G. J., Kuczera, M., Mintenig, S., Lorenz, C., Gerdts, G. Focal plane array detector-based micro-Fourier-transform infrared imaging for the analysis of microplastics in environmental samples. Environmental Chemistry. 12(5), 563-581, doi: http://dx.doi.org/10.1071/EN14205 (2015).
23. Corcoran, P. L., Biesinger, M. C., Grifi, M. Plastics and beaches: A degrading relationship. Marine Pollution Bulletin. 58(1), 80-84, doi: 10.1016/j.marpolbul.2008.08.022 (2009).
24. Ioakeimidis, C., et al. The degradation potential of PET bottles in the marine environment: An ATR-FTIR based approach. Scientific reports. 6, 23501, doi: 10.1038/srep23501 (2016).
25. McDermid, K.J., McMullen, T.L. Quantitative analysis of small-plastic debris on beaches in the Hawaiian archipelago. Marine pollution bulletin. 48, (7), 790-794, doi: 10.1016/j.marpolbul.2003.10.017 (2004).
26. Eriksen, M., et al. Microplastic pollution in the surface waters of the Laurentian Great Lakes. Marine pollution bulletin. 77(1-2), 177-182, doi: 10.1016/j.marpolbul.2013.10.007 (2013).

Figure

Click here to download Figure Figure 1.jpg

Figure

Click here to download Figure Figure 2.jpg



Figure

Click here to download Figure Figure 3.jpg

Figure

Click here to download Figure Figure 4.jpg

Figure

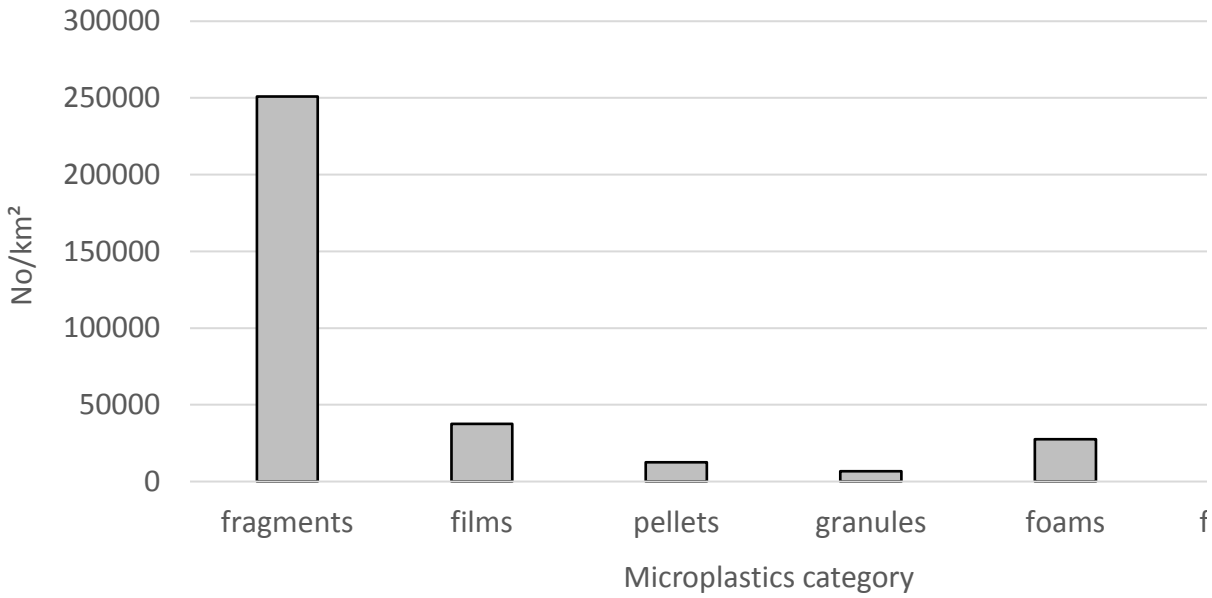
Click here to download Figure Figure 5.jpg

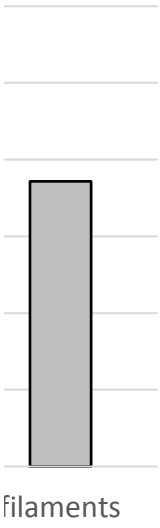
Figure

Click here to download Figure Figure 6.jpg

Figure

Click here to download Figure Figure 7.xlsx

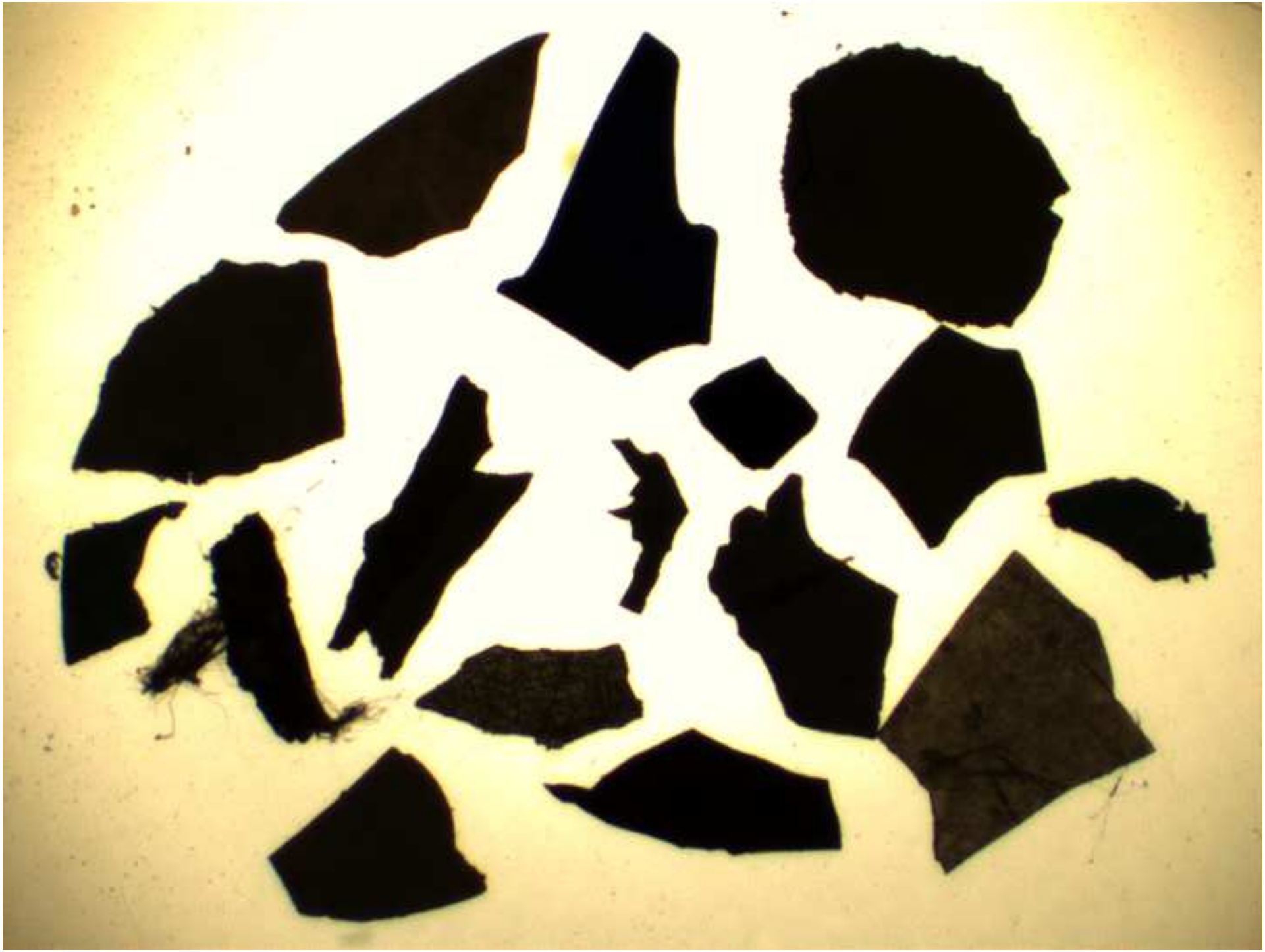




	No	No/km2
fragments	301	250833
films	45	37500
pellets	15	12500
granules	8	6667
foams	33	27500
filaments	223	185833

Figure

Click here to download Figure Figure 8a.tif

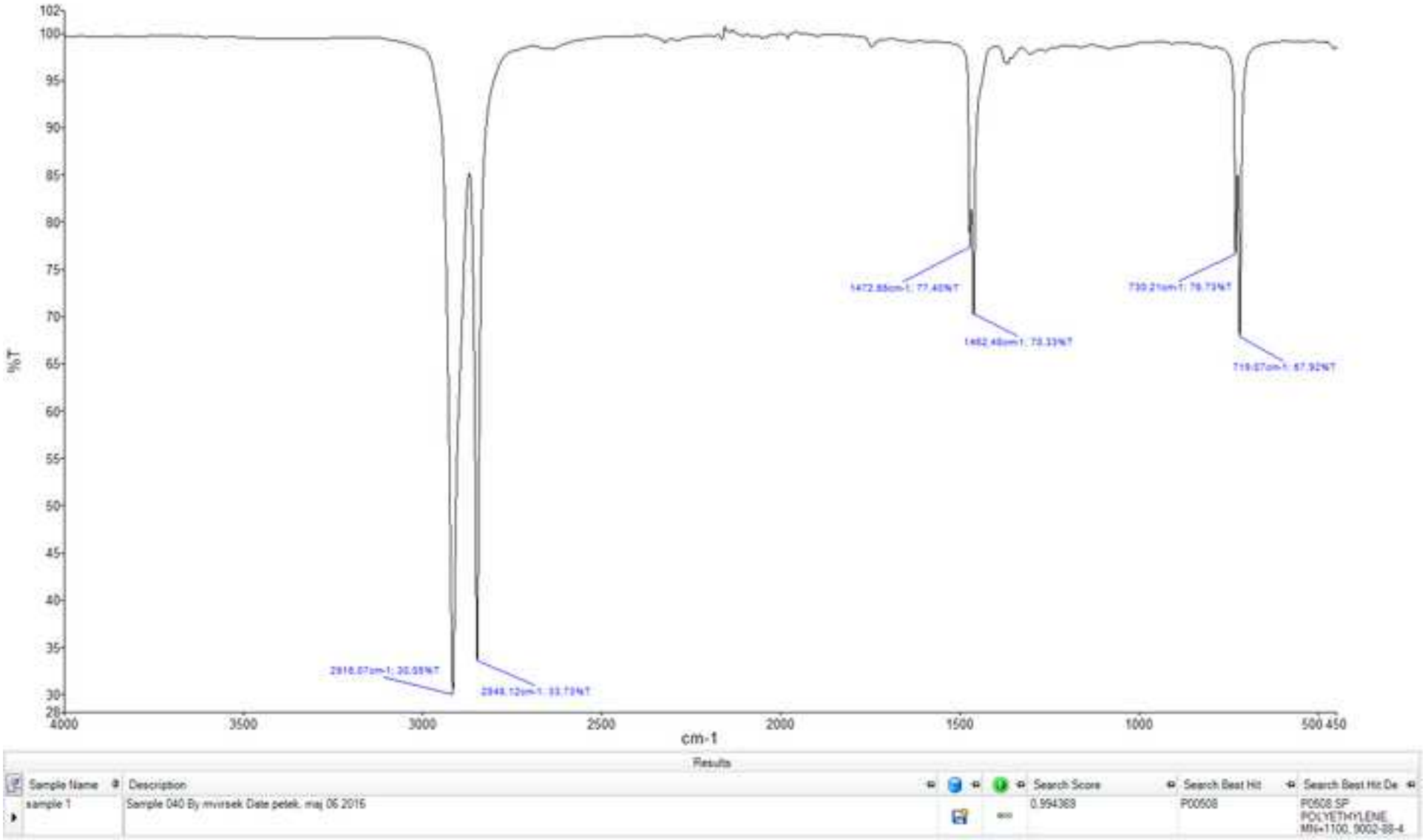


Figure

Click here to download Figure Figure 8b.JPG

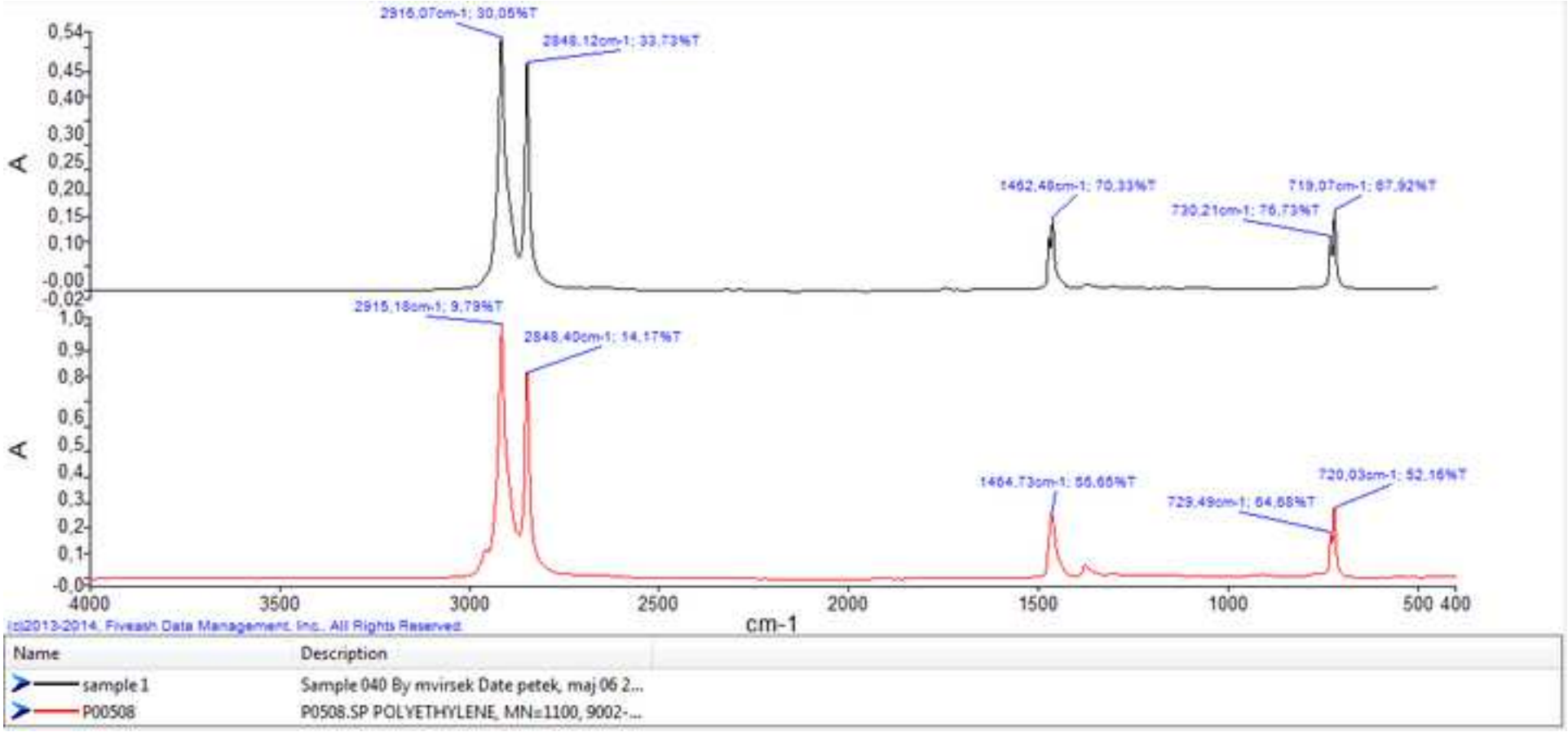
Figure

Click here to download Figure Figure 9.png



Figure

Click here to download Figure Figure 10.png



Table

Click here to download Figure Table 1.xlsx

1	Fragments
2	Films
3	Pellets
4	Granules
5	Filaments
6	Foams

Table

Sampling distance [km]	2
Manta width [km]	0.0006
Sampling area [km^2]	0.0012

Table

Click here to download Figure Table 3.xlsx

	No	No/km^2
fragments	301	250833
films	45	37500
pellets	15	12500
granules	8	6667
foams	33	27500
filaments	223	185833

Table

Click here to download Figure Table 4.xlsx

Index Region	Area [mm²]	Maximum length [mm]
1	8.010	5.506
2	10.517	5.628
3	12.185	5.429
4	3.367	3.367
5	2.475	2.155
6	1.809	2.943
7	6.604	5.238
8	5.779	4.037
9	4.472	3.791
10	16.907	5.355
11	7.246	3.733
12	7.867	4.622
13	6.411	5.056
14	3.281	3.070
15	12.937	5.554
16	6.709	3.716

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment JoVE Materials.xls

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
In this protocol no specific equipment or reagents were used.			

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Protocol for microplastics sampling on the sea surface and sample analysis

Author(s): Kovač Viršek, M., Palatinus A., Koren Š., Peterlin M., Horvat P., Kržan A.

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	KOVAČ VIRSEK, MANCA
Department:	SECTOR FOR MARINE WATERS
Institution:	INSTITUTE FOR WATER OF THE REPUBLIC OF SLOVENIA
Article Title:	PROTOCOL FOR MICROPLASTICS SAMPLING ON THE SEA SURFACE AND SAMPLE ANALYSIS
Signature:	
Date:	24.6.2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Response letter to the manuscript JoVE55161 "Protocol for microplastic sampling on the sea surface and sample analysis"

Editorial comments:

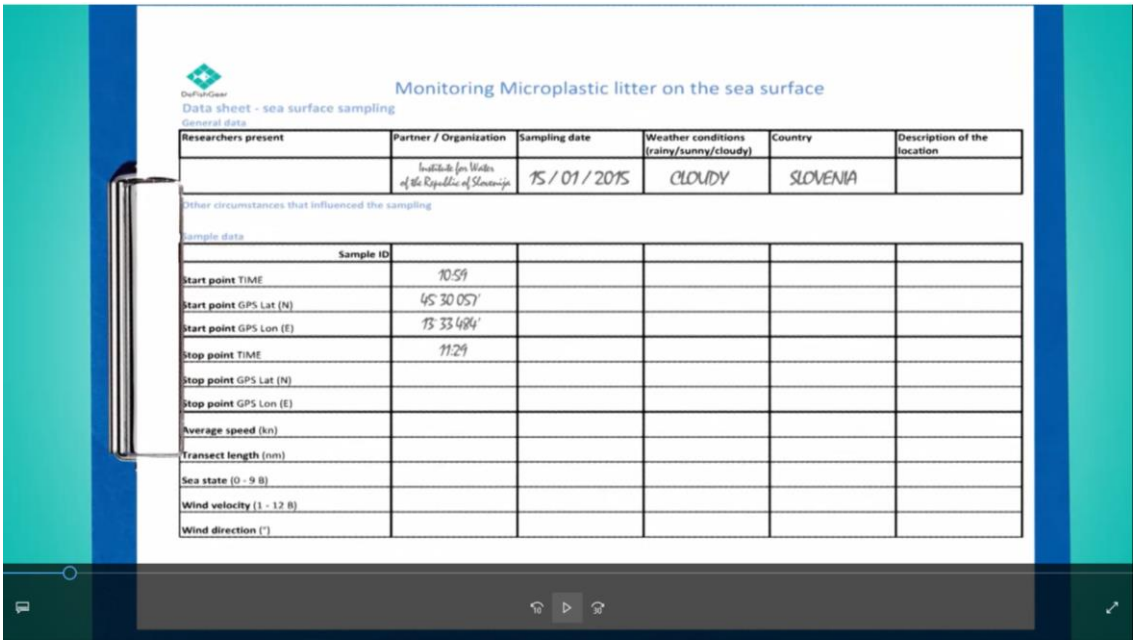
"Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Graphics/screen capture issues

• 0:33, 0:50 - When the video is scaled to our webplayer's size, the text in this graphic is very difficult to read clearly. I would recommend scaling the graphic up or making the text larger."

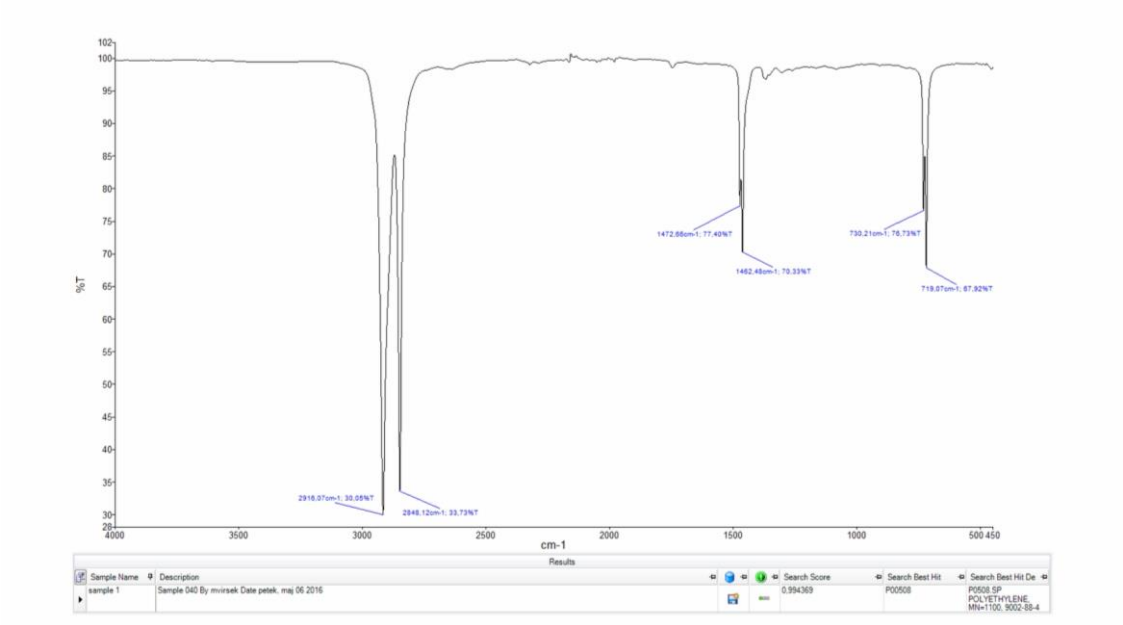
Answer: We do not know where exactly is the problem because on our side the resolution of these pictures is sufficient to clearly read the data (see picture bellow, Prt Scr from our computer).

Actually, the spreadsheet is only an example of a spreadsheet, the labels in the fields are not crucial for the understanding of the protocol. Each researcher should prepare his/her own spreadsheet for the field work. The only important thing is, that data such as the date, start and stop GPS coordinates of the transect and wind speed are noted as this is mention in the text (audio and manuscript).



• 9:17, 9:31- If any of the text in these figures is important for the viewer to see, it should be enlarged.

Answer: The labels of the peaks are not important for the viewers, since we are not talking about specific peak wavelengths, but about general spectra that is acquired from the sample and later compared with the spectral library.



•Audio issues

• *1:17 - It sounds like the diegetic sound was left in for this shot. The sound should be removed for clarity and consistency.*

Answer: We think that the diegetic sound is not disturbing the viewers' concentration since the sound in not loud and does not interfere with the narrator voice. We showed the video to several people, and no one mentioned that this part would be disturbing the clarity of the speech and the protocol**.**

• *If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.*

Answer: All the figures and tables are made by authors or video producer.

• *JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.*

Answer: We checked the references again and added the DOIs where they were available**.**

•*IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.*

Answer: Before submission the manuscript was edited by native English speaker (from USA). The language and grammar will be checked again prior to the resubmission.

Reviewers` comments:

Reviewer #1:

Manuscript Summary:

Comments on JoVE55161 by Kovač Viršek et al. (see also attached manuscript R_1_annotated):

Merits of the study: This manuscript describes an instructional video for microplastic sampling. This is very helpful because there are more and more people starting to sample microplastics and it is very important that they all work in the same way. This video can help here. However, there are a number of things that can be improved.

1) Title: ok

2) Abstract: ok

3) Introductory part: ok, motivates the study well.

4) Protocol: ok, but there is confusion with the numbers. Also, there are some steps that are not clearly emphasized neither in the written nor spoken text, but that obviously do happen - for example, no mentioning is made of the second label that is placed in the jar, but in one video-sequence, this label can clearly be seen.

Also, some parts that are important for the methodology, e.g. using a flowmeter, are not shown in the protocol or video, even though they are very important! This description is first mentioned in the discussion section, but should be part of the protocol.

Also, the lab control should be mentioned here.

5) Representative results: ok, but the figures and tables need captions! Some of the tables look as if they were copied straight out of excel without any editing work! These should be improved.

6) Discussion: the discussion is a bit confusing and in some sections not really a true discussion, but instead reads like a protocol description. Some of these sections (e.g. the lab control, or the flowmeter section), if they are maintained here in the discussion section, will need references, which are out there and the authors should search for them.

7) Literature cited: I have not checked whether all papers are cited in the text and viceversa.

In summary, this video and the related text can become very useful documents in the future, but they require careful revision before they should be published in the journal.

There are a number of additional comments in the annotated manuscript. In case there are any questions about our comments, we invite the authors to contact us directly.

Answer: We thank the Reviewer #1 for the thorough review of the manuscript and the video, and all the valuable comments. We took all the given comments into consideration and replied on the reviewer's comments bellow in the response letter and made correction in the manuscript. Therefore we explained the confusion with numbers which emerged after editing by the journal editorial office

of the manuscript. We proposed that in order to provide logical sequence and content of the steps in the protocol, the original enumeration and division of paragraphs should be accepted.

We added into the protocol a suggestion about the second label of the samples.

Our protocol is describing the sampling without flowmeter, therefore we mentioned the use of flowmeter first in the discussion as an additional option. The recommendation of using controls during laboratory analyses was already included into the submitted manuscript (lines 357-360).

We do not understand the comment 5, that »figures and tables need captions«, since there are captions above figures and tables in the manuscript and also in the video protocol. The tables weren't made in the Excel. The comment is very biased, since the editors didn't have any comments on the quality of our tables. Therefore we didn't consider this comment as necessary to abide.

The discussion was written by the document »Manuscript Instructions for Authors« from Jove. The Editors did some changes in splitting the paragraphs into several (there were 5 before, and after the editing there were 7). We suggested the correction of one of the paragraphs to be merged in different way than was suggested by the editors.

As already mentioned, the mention of flowmeter will stay in the discussion, and the references were added. The mention of lab control was included in the discussion and is appropriately referenced.

All the literature cited in the text is listed in the »References« section of the manuscript.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Manuscript comments (Reviewer 1):

Comments to introduction:

Comment A1, line 71: »*References needed here*«

Answer: Reference has been added.

Comment A2, line 73: »*References needed here*«

Answer: Reference has been added.

Comment A3, line 74: »*The citation numbering is wrong here and throughout he whole manuscript.*«

Answer: Numbering of references have been corrected according to the JoVE manuscript instructions for authors.

Comment A4, line 75: »*Microplastics are not only circular.*«

Answer: The word “diameter” was deleted.

Comment A5, line 76,77: »*You describe below that the mesh size used during the monitoring is 300µm, which therefore should be the lower limit of the microplastics analyzed with the present method. I think it is important to also standardize the lower limit of the microplastic analyzed.*«

Answer: Yes, we completely agree that also the lower limit of the microplastic has to be defined in the definition of the term microplastic. Now it is assumed that the low limit is the mesh size of the sampling net. As we did not find any new definition of microplastic where the lower limit is defined, we did not make any modification in the manuscript.

Comment A6, line 81: »*This sentence is not clear. Pleaser rephrase*«

Answer: Sentence has been corrected.

Comment A7, line 83: »*References needed here« and* ***Comment A8, line 83****: »How do sea currents break down microplastics? I think breakdown through contact, collision with other fragments or floating objects and abrasion is meant here.*«

Answer: Sentence has been corrected and reference has been added.

Comment A9, line 91: »*The verb here should refer to 'the small size of the microplastics', which is the subject of the sentence. However, I guess the authors wanted to write here that the microplastics are consumed*«

Answer: Sentence has been corrected to be more understandable.

Comment A10, line 94: “*This is mainly the hydrophobic surface of the microplastics which allows POPs to fix. Ratio surface/volume enhance that effect.*”

Answer: In the sentence also the term “hydrophobic surface” has been added.

Comment A11, line 96: “*But the POPs come from the environment. I think the authors here are talking about the additives part of the composition of the microplastics*.”

Answer: Sentence has been corrected to be more understandable.

Comment A12, line 98: "*References needed here*."

Answer: Reference has been added.

Comment A13, line 104: " *This study is useful to provide a standardize method for microplastic monitoring, not only in the EU seas but also worldwide. This argument should be better developped here to justify the importance of the present study*.«

Answer: Sentence has been corrected.

Comment A14, line 112: "*Where does this claim come from. References are needed here*."

Answer: Reference has been added.

Comment A15, line 116: "*I do not understand the logic of the argumentation here. I recon that that part belongs to the protocol section below*.«

Answer: In this part of the introduction, the short descripton of our work is described. This paragraph has been corrected to be more understandable.

Answers to Methodology

Comment A16, line 123: *I think it could also be interesting to describe here the method in case small vessel do not have a spinnaker boom on the side. In that case, the trawl should be dragged ~50 m from the stern of the boat to reduce turbulences of the sea surface generated by the boat propeller and reduce contamination from the boat.*

Answer: This protocol describes the sampling with a use of spinnaker or »A-frame« from a side of the vessel. For dragging a trawl behind a vessel, a separate protocol would be in place. We mentioned this option in discussion with 2 references.

For use of trawl, dragged from the stern of the boat, 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 boat.

Comment A17, line 128: *The GPS coordinates only allow to calculate the area filtered. However, I realized from my own monitoring that the actual volume of water filtered during the sampling can vary greatly depending on the courant. This is why I think it is crucial to fix a flowmeter at the entrance of the mouth of the trawl to measure the volume of water filtered during the sample. I strongly recomment to add this to the method.*

Answer: The use of a flowmeter is a welcome addition to the method, but this protocol describes the sampling without the flowmeter and mention of the possibility of adding one is emphasized in the discussion. (See also answer to the Comment A50)

Comment A18, line 130: *Based on the transects we did in the bay here recently 3 knots was already too fast for the neuston net, but it may be fine for a manta net, I don't know.*

The manta net is firm and stable enough that the sampling at speed up to 3 knots, fixed from the side of the vessel, is possible without any problems. During DeFishGear project, 44 transects of sea surface were sampled two times by 6 different groups of experts, with speeds from 2 to 3 knots, and none of the expert reported any problems.

Comment A19, line135: *Most modern GPS devices also record the transect. If it cannot be guaranteed that the boat sampled a straight transect (for example rougher conditions) the distance could be calculated more reliably through this.*

Answer: We added into the protocol, that the most correct way to calculate the length of the sampled rout is from GPS coordinates.

Comment A20, line 152: *Should this read ''at least 70%''? (as said in the video)*

Answer: No, it was meant "70% ethanol", since we prepared exactly 70% ethanol that is standard for the use in laboratory. And in the video it is not said *''at least 70%''*.

Comment A21, line 155: *There is a risk that the ink of the marker be washed away by the alcohol used to preserve the sample. I recommand to label the samples using masking tape and pencil.*

and

Comment A22, line 155: *And in addition you should put a pencil-written label (or previously printed with laser printer) inside the sampling jar!!! You should always have two labels, one inside the jar, and one on the outside of the jar!!!*

Answer: We improve the protocol with additional backup label.

Comment A23, line 161: *No capital letter after opening parentheses*

Answer: Corrected.

Comment A24, line 162: *So then why not reducing the sampling time to 20 min to provide more general method. I personnaly sample for 20 min due to high organic matter along the coast of Chile. Also, many studies sampled for 20 min (Gago et al. 2015,).*

Answer: This protocol was developed on the basis of the document »Guidance on monitoring of marine litter in European Seas.", provided by Marine Strategy Framework Directive Technical Subgroup on Marine Litter (Hanke, 2013), where Institute for Water of the Republic of Slovenia is also a member. And in this document the proposed sampling time is 30 min. Furthermore in our experiences after 20 minutes manta net was never clogged, so we always measured for 30 minutes to get better

representative sample. We also mentioned that if you have any problems, you should adapt the sampling time.

Comment A25, line 162: *More to avoid contamination onboard the vessel: Make sure to not drag the net or the rope to deploy the net across the vessel. Avoid contact of net with the boat hull while deploying and capturing.*

Answer: We added this comment as additional notes under "NOTE: General sampling conditions".

Comment A26, line 167: *Ok, but where do you put this? And will you also count this?*

And

Comment A27, line 171 : *Does this refer to the objects from the previous paragraph? Why do you now further determine all objects > 25mm?? Is this identical to the video?*

Answer: The editors made a new paragraph in a very unfortunate place, so that now both paragraphs are confusing. We joined them back together, because we think that it is important that those two steps stays together as one, since this step is all about meso and macro litter, and there is no need to devote more space in the protocol for this as this is an optional step.

Comment A28, line 173: *It would be necessary to measure the objects here already. Is this desired? If so, it should be added.*

Answer: This is not intended and these data are not important as we talk about microplastic and not macro litter.

Comment A29, line 173: *This section is confusing – also in the video it is confusing, because while you are talking about ">25 mm", the object that is being manipulated is clearly <25mm!!!*

Answer: As mentioned in the answer on comments A26 and A27 – this step of the protocol is one step and in the video the entire step is demonstrated, in this case the particle in the video is smaller than 25 mm but larger than 5 mm, therefore meso litter that is mentioned in this step.

Comment A30, line 181: *Please give an order of the maximum volume of each subsample that should be taken.*

Answer: We think that there is no need to define the size of the subsample, since the size of the sample depends on the amount of organic and inorganic (natural) and plastic material in the sample, also in the different laboratories Petri dishes of different sizes are used. In addition, the size of the subsample should also reflect the level of experience for recognizing the microplastic of the person examining the sample. Also it is up to the researcher how much of the total sample collected they will examine.

Comment A31, line 185: *Further: 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. I work with a magnification between 16x and 20x to search for plastics and more to identify them (searching for plastics with 80x sounds very tedious).*

Answer: Comments have been added in notes in the step 2.5.

We are using magnifications from 20x to 80x all the time, 20x for general examination of the sample and larger magnifications to zoom in on specific particles to check the characteristics of the specific particle.

A32, line 192: *What if this is not possible? How to proceed? Possibly give a confidence rating, e.g. 1 = very sure that present item is plastic, 2 = not sure, 3 = probably no plastic? Any suggestion?*

Answer: This protocol describes the use of chemical characterization in the end as we believe that results are reliable only if identification of chemical structure is performed for the particles. The proposed method with the use of confidence rating would be very subjective. It is better to use polarization light on the microscope to distinguish between plastic and non-plastic material.

A33, line 194: *What could this be? We use a piece of a plastic mesh with a known mesh size as a scale to take pictures.*

Answer: Measuring equipment of a microscope means an ocular ruler calibrated by the micrometer slide for the microscope or image analysis software. The protocol was improved.

A34, line 195: *I think the shape and erosion of the microplastics are also important characteristics that should be described (see Table 6 in Hidalgo-Ruz et al. 2012).*

- Shape: sharp-edged fragments may lacerate the digestive tract tissues.

- Erosion: degraded fragments may release additive more easily than newer particles (Andrady 2011).

Answer: This protocol describes simple characterization of the microparticles for their size and color. We do not think that for general estimation of marine environment pollution with microplastic particles the shape of the particles and degree of erosion are key information.

A35, line 200: *Is this commonly done and feasible? What precision would the scale have to have?*

Answer: It is feasible in our experiences to weigh all the particles from one category on analytical scale with four decimal places of precision.

A36, line 201: *How do you make sure then that the sample will not get contamination from the dessicator?*

Answer: The protocol was corrected as “closed Petri dish”. This was our mistake.

A37, line 201: *Also for 24H? This is not clear from that sentence*

Answer: The protocol was corrected.

A38, line 204: *Something seems to go wrong with the enumeration here, which is jumping all over the place.*

Answer: Enumeration was checked and corrected where this was needed.

A39, line 208: *This is why I beleive geenral shape of the microplastic should be described (see comment [NO21])*

Answer: As already mentioned in the answer on the comment A34: We do not think that for general estimation of marine environment pollution with microplastic particles, the shape of the particles are key information, recognising the additional features of plastic particles only helps at faster recognition.

A40, line 208: *Alcohol helps here because it discolours the organisms and colorful plastics therefore become easier to see. Possibly add this?*

Answer: The protocol was improved for one additional note in the step 2.2.

A41, line 208: *Further signs that something is not a plastic: transparent pieces that break very easily (possibly parts of crustacea or pteropds)*

Answer: We had experiences with fragile and transparent pieces that were determined as plastic by chemical characterization. In our experience these are most commonly (but not exclusively) highly degraded foils. Therefore, it is “dangerous” to make a statement from which people would conclude that all microparticles that are transparent and fragile at the same time are always parts of exoskeleton of crustacean or pteropods.

It is true that researchers with little experience with work by stereomicroscope and biological samples, can confuse carapax for plastic, but if you use bigger zoom and polarization light, carapax is easily recognized in the sample.

Comments to Results:

Comment A42, line 254: “Unclear. Please rephrase.«

Answer: The natural colours have been added.

Comment A43, line 259: I do ot understand this sentence. Main result of what? Please clarify.

Answer: Sentence has been corrected to be more understandable.

Comment A44, line 259: “Data is the plural form: one datum, many data!!«

Answer: Correction was made.

Comment A45, line 265: “By the width of the net opening.”

Answer: Correction was made.

Comment A46, line 266: “For this it would be necessary to take pictures of the plastics. I don't think this is mentioned in the protocol.”

Answer: In the protocol this step (to take the picture for image analysis) is not mention, because this is the specifity of the image analyis software that researcher use it. For instance, we work by AxioVision (Zeiss) software, but we did not mention it in the protocol, while there are some specific rules from JoVE (below).

“Style Guidelines:

Avoid the use of commercial language, including any TM/R symbols or the mention of company brand names before an instrument or reagent

TABLE OF SPECIFIC MATERIALS/EQUIPMENT:

This table should include information for viewers to obtain the materials used in the protocol such as company, web address and catalog number. It is appropriate to include the specific brand of reagent used in your experiments especially if this specificity influences the outcome of the experiment. However, it is not appropriate to mention specific brand names or company names throughout the manuscript text. Please avoid the use of any copyright or trademark symbols throughout the text, especially in the tables of specific reagents and equipment.”

Answers to the Discussion:

Comment A47, line 324: *This chapter s long, repetitve, poorly written and does not have any subchapter. Please, improve the English and shorten*.

and **Comment A48, line 324**: *In most parts of the discussion, you are basically repeating what has already been written in the method. I recommend to substantially reduce the length of the text and make better use of the litterature to support your argumentation. Also, various aspect of the methodology are mentioned for the first time in the discussion (see comments below), although they should belong to the Method section.*

And **Comment A54, line 346:** *All this does not sound like a »discussion« section, but rather like a Material&Methods section.*

Answer: This chapter is written according to the JoVE instructions for authors (see below), where methodology have to be disscussed.

“DISCUSSION: (3-6 paragraphs)

Please remember that JoVE articles are focused on the method and protocol. Thus, the discussion should be similarly focused. This should be written in full sentences and paragraph form. This section should discuss:

• *Critical steps within the protocol*

• *Modifications and troubleshooting*

• *Limitations of the technique*

• *Significance of the technique with respect to existing/alternative methods*

• *Future applications or directions after mastering this technique"*

Comment A49, line 329: "*Wording is strange – please, rewrite*."

Answer: Sentence has been corrected.

Comment A50, line 337: "*The use of a flowmeter is not described in the method. I think you need to improve the structure of the text to make it more easy to follow. That part should definitely be mentioned before.*"

And

Comment A51, line 337: "*It also seems very relevant to include this in the video. You probably could also include the equations that you are using in the video! Is the step of recording the station of initiation of the trawl and the end of the trawl, also in the video? If not, then maybe add?!?*"

Answer: The use of a flowmeter is not described in the method, because we did not use them when video was produced. There are a lot of studies, where flowmeter was not used and flow meter is just one of options that could be used if the researcher have opportunity to use them. For this reason we mention in discussion option to use the flowmeter and that the use of flowmeter could be one of the improvement of the protocol.

The step of recording the station of initiation of the trawl and the end of the trawl is already in the video.

One correction was made in the text, to make it more easily to follow.

Comment A52, line 340: "*Is this not very fast?!"*

Answer: Depends of the sea. For this reason range (2 – 3 knots) was proposed and factors on which you have to be careful were mentioned. See also the answer to the Comment A18.

Comment A53, line 341: "*You mentionned that wind speed should always be <2 Beaufort. This need to be clarified.*"

Answer: The clarification was made in the NOTE at the end of the first paragraph of the protocol (Sampling of microplastic on the sea surface).

Comment A55, line 341: "*References neede here*«

Answer: Reference has been added.

Comment A56, line 352: "*This also has not been mentionned in the method. Discussion should only focuss on what has been described earlier in the text*.«

Answer: This was not mentioned in the methods, while the separation of microplastic from the sample could also be done by the use of stereomicroscope without polarization light. This is just one option that can help researcher to find microplastic particles in the sample. And this is discussed in this chapter, according to the instructions of JoVE (see answer to the comments A47, A48 and A54).

Comment A57, line 354: "*Really? Particles > 0.5 mm can not be identified with FTIR spectroscopy?? It seems that this should entirely be possible*!"

Answer: »Particles > 0.5 mm can not be identified by FTIR spectroscopy« - this is not written in the text! In the text is written that: »particles >0.5 mm can only be identified visually, by the use of stereomicroscope«, but particles <0.5 mm have to be verified by the use of FT-IR microscope. But of course if you have option to make the chemical analysis also for the particles >0.5 mm, your results are more reliable.

Comment A58, line 362: "*Was this investigated somewhere or is this a hypothesis? For example, I'm not sure if the magnification (beyond ~20x) of the stereomicroscope is one of the most important criteria here.*«

Answer: This was written on the basis of our experiences and experiences of other scientists (e.g. Lőder and Gerdts, 2015). From our experience the quality of the microscope is one of the most important factor that influence on the results (the source of the light, the quality of lens). See also answer to the Comment A31.

Comment A59, line 364: "*As this appears in the discussion section, there should be some references here! There are several studies out there, where people did use these controls and you should refer to those!*"

Answer: Reference has been added.

Comment A60, line 370: "Again, this not has been mentionned before in the method. Here you could have discussed the possibility to not take in account fibers when clean air flow chambers are not available, as described in the OSPAR request on development of a common monitoring protocol for plastic particles in fish stomachs and selected shellfish on the basis of existing fish disease surveys.«

Answer: See answer to the comments A47, A48 and A54.

Comment A61, line 370: »Yes, but the equipment is expensive!«

Answer: The quality of the results can be in most cases also depended of the equipment used in the study.

Comment A62, line 394: “General comment about this writing of »microplastic separation« and other similar expressions – i am not a native speaker, but it is my impression that the noun »microplastic« is used as an adjective for the noun »separation« - if that is the case, then »microplastic« should always be in singular form! Check with a native speaker and if necessary, correct throughout the entire document!«

Answer: The phrase “microplastic separation” was replaced by the phrase “separation of microplastic”.

Reviewer #2:

Manuscript Summary:

First and foremost, I would like to thank the authors for the insight in addressing microplastic pollution on a methodological perspective.

I believe this is a very significant work concerning the worldwide need for standardised protocols, and as someone working on the same research field; I use this opportunity to thank once again the authors.

My only comment concerns micro-FTIR techniques. I agree with everything the authors wrote, from pre-treatment of microplastic particles with 30% H2O2 when necessary, to background spectra to reduce noise, but I would also wanted to share with the authors, that sometimes there is apparent noise in the spectrum collected with is likely to be linked to polymer degradation throughout time. Fragmentation does not interfere with chemical structures, but degradation can, and that is likely to somehow affect some of the characteristic bands of some polymers. Please take this information into account. For more on this, please consult manuscripts from conservation and restoration of art pieces, from the 1970's onwards as usually these chemists have a great knowledge on polymer science and FTIR spectra analysis.

After everything said, I find the manuscript quite well written and suitable for publication as it is.

Answer: We thank the Reviewer #2 for the thorough review of the manuscript and the video and for all the compliments.

We took the comment about the mention of degradation of the plastic as possible reason for the noise in the FT-IR spectra in consideration. We didn't change the discussion about degradation of microplastics, since it is already mentioned (lines 359-360), that with (in-depth) analysis of the FTIR spectra, an additional information on particles, such are oxidation and level of degradation, can be acquired.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Authors comments to the editors:

- We noticed the formatting changes that you made in the numbering (in the protocol) and paragraphs (in the discussion) of the manuscript. We cannot agree with these corrections. They were made in unfortunate places interfering with the understanding of the protocol. Reviewer #1 also commented that »there is confusion with the numbers« (in the protocol). We therefore suggest if we can use the original numbering of the protocol, since it was placed in order of logical steps. Step 2.2 shouldn't be separated into two steps, since it is an optional step and we don't want to disturb the reader with several steps he/she should over skip. In the discussion we made a new paragraph, but in a different place, where, we believe, it is more suitable.