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# The use of fluorescence methods for identification of microplastics:

## the case of the Metaponto beach (Basilicata, Italy)

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# ABSTRACT

Microplastic (MP) pollution in the natural environment is currently the subject of growing interest both for the scientific community and for the World Health Organization. This interest is confirmed by hundreds of scientific articles published every year. The growing attention to this type of contamination in the various environmental matrices and, consequently, of living organisms, is caused by the following factors: increase of production and consumption of plastic, relatively small recycling rate of synthetic polymers at the end of life (only between about 9 and 15% is recycled from about 400 million tons of plastic produced annually worldwide), and the inability of current treatment plants to treat this type of waste, especially as regards textile fibers that are difficult to estimate.

The purpose of this thesis is to evaluate the efficiency of expeditious optical microscopy techniques and the related sample preparation techniques for the recognition of MPs in marine sediments. The analyzed samples were taken with appropriate sampling from the coast of Metaponto, a city located in Basilicata (Ionian coast, southern Italy), to detect the presence of this type of pollutant. The experimental research was carried out using electrostatic and densimetric separation methods, using for the last one a NaCl solution. The identification and counting of MPs have been made by optical microscope with UV flashlight both in the absence and in the presence of Nile Red dye, which currently appears to be one of the least studied identification techniques, and which was mainly used in the field of biology and medicine (and related fields) until 2010, but not in the field of ecology.
It was possible to check the efficiency of the electrostatic separator in order to reduce the sample volume and increase the concentration of MPs contained in it. In general, among the analyzed MPs, the most common size range was found to be those <0.5 mm. Meanwhile, synthetic fibers, transparent under LED, and fluorescent under UV light, were found to be the predominant ones. From identification under the microscope, they appear to be on average 1.41 MPs/g. This result is comparable to the one obtained after staining with Nile Red, which is equal to 1.56 MPs/g. From further analysis of the examined sample, however, despite the similar numbers, in half of the cases, the Nile Red colored other objects and not those that were identified through the microscope as MPs. Therefore, a further study of the combination of these technologies is necessary with the aid of spectroscopic techniques (FT-IR, Raman), which are capable to provide the chemical composition of the material under examination.

The analysis of numerous scientific articles on the subject of MPs, carried out during the experimental research, also with the aim of refining and improving the methods of followed investigation, led to finding of some contradictions in the results of the studies published so far. This fact leads to further confusion in a research field, which is already quite complex and multifaceted, and which currently does not have standard protocols. One of the major causes of errors and uncertainties in MPs recognition and counting is visual identification using the UV light microscope, which can lead to both overestimating and underestimating the amount of MPs, if a researcher relies on this method alone and did not combine it with other more reliable ones, especially when the threshold of less than 0.5 mm is exceeded, which is precisely the most frequently detected dimension.

# INDEX

# 1. PLASTIC POLLUTION: general overview

In the early 1960s, Swedish engineer and creator - Sten Gustaf Thulin, has conceived plastic bags and packaging as a panacea in solving the problem of increasing deforestation for the production of packaging and other purposes, since this type of packaging was short-lived and quickly became unusable, and recycling was then only in its infancy (recycling of paper and paperboard products increased from approximately 5 million tons to 44 million tons, from 1960 to 2017 according to the Environmental Protection Agency (EPA) of the United States). After more than half a century, the problem of cutting down trees has not gone away, and the durability of plastic has turned against nature and humanity, since the creator of plastic did not take into account the factor of the human nature of behavior and what scales the world level of consumption and indifference to environmental pollution can reach both on the part of the consumer and the manufacturer, in pursuit of production optimization and cost reduction (at the cost of the same plastic recycling). Now plastic is everywhere and in huge, ever-increasing quantities (***Fig**. **1.1***), and its diversity, that is, the variability of the chemical composition, leads to even more complex problems than just quantity, namely, the inability to unify the processing for all types, or even its impossibility (as is the case with thermoplastics), as well as different behavior and impact when released into the environment.

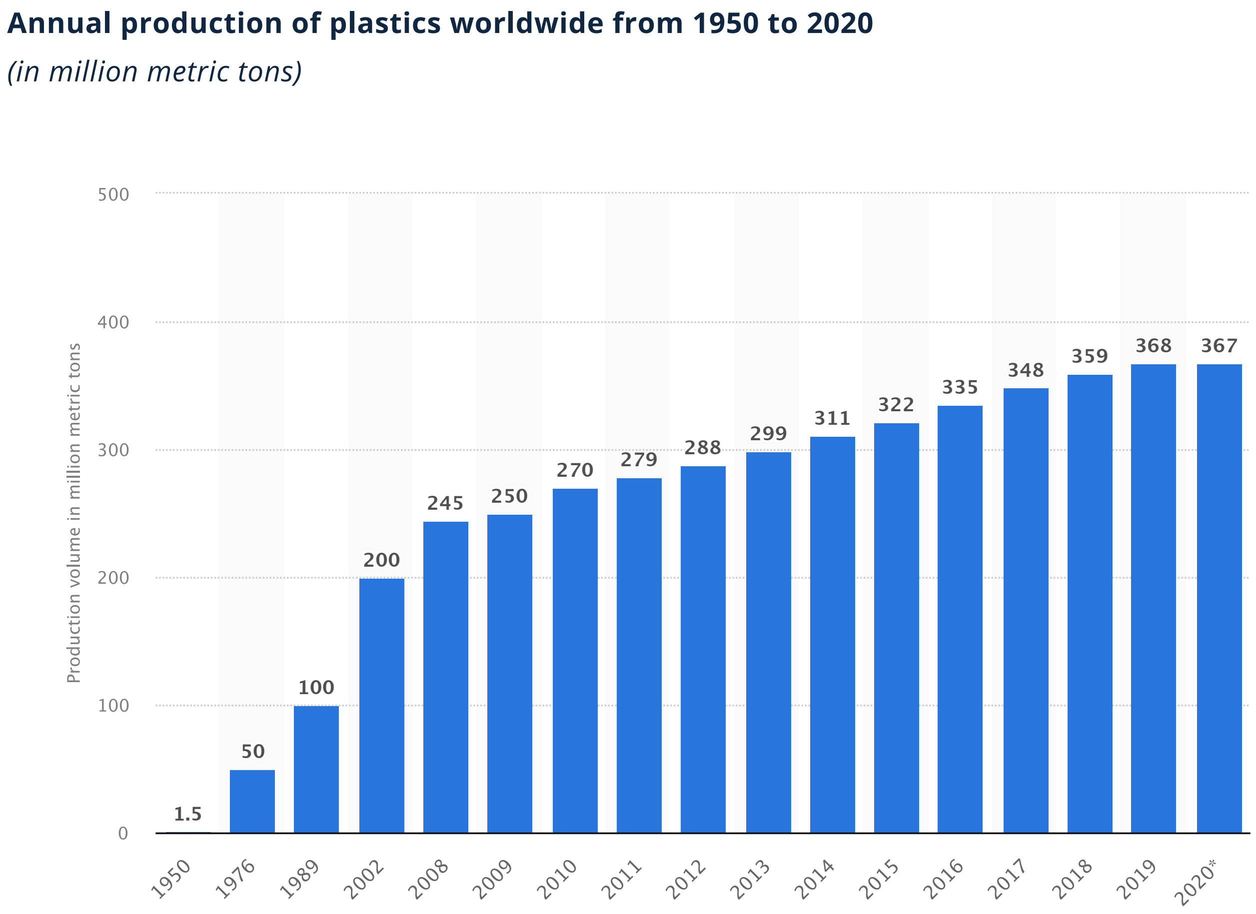

**Annual production of plastics worldwide from 1950 to 2020**
*(in million metric tons)*

***Fig**. **1.1.** Statistical data of the increase in plastic production (font: statista.com)*

The problem of plastic pollution, which attracts the attention of the world, is extremely complex, as any problem that simultaneously involves both the global and the local scale, and as well as combines the inevitable technological progress (for instance, 3D Printing which utilizes plastic materials more and more for the production of different complex objects) and the habits, modern lifestyles of people i.e. the multifaceted social aspect as consumption and length of life of plastic items. Often, it is the low cost of plastic, as a result of improving the chemical composition and production technology, that makes it possible not to think about reuse or recycling. In this connection, the production of plastic from secondary raw materials in most cases is uncompetitive comparing to the use of primary raw materials as long as bioplastics. Therefore, they need to be stimulated (legally and/or financially) by United States, as well as environmental awareness and activity of every person who can properly dispose of certain items, reduce the consumption of plastic, and also prefer to purchase more expensive material made from recycled materials.

## PLASTICS AND MICROPLASTICS

Denomination "plastics" includes all synthetic polymers formed by the fusion of two to several thousand simple molecules (monomers), resulting in a large molecular structure, which may result in such main groups of plastics as: acrylics, polyesters, silicones, polyurethanes, and halogenated plastics. Various additives such as pigments, plasticizers, light, heat and thermal stabilizers, etc. can also be added to these materials, affecting the final chemical and physical properties of the plastics. As a result, despite the variety of synthetic polymers, the most frequently used materials throughout the year are the ones shown in ***Fig. 1.2.***

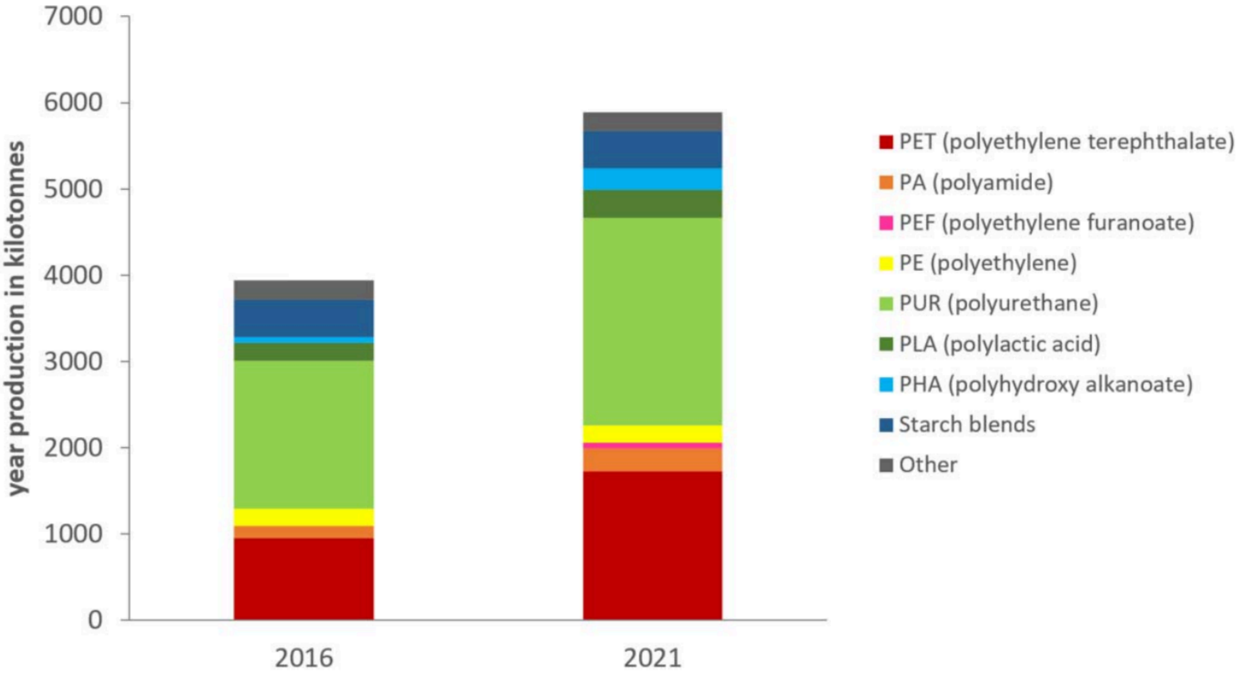


***Fig. 1.2****.Global production capacities of bio-based plastics in 2016 and estimations for 2021 (source: Alaerts et al., Sustainability 2018, 10, 1487)*

One of the main properties of plastics is their durability due to poor biodegradability. For instance, it takes 450 years for a plastic bottle to decompose in average. One such bottle consists of different plastic materials with different properties. Thus, the amount of time required for this process depends on the type of plastic and the environment in which it is placed (***Fig. 1.3***). However, thanks to various programs such as the European Green Deal (as part of a strategy to achieve the Sustainable Development Goals (SDGs)), there is a trend to replace some types of classic plastics with bioplastics (***Fig. 1.4***), which are capable of faster laying (up to six months as a rule) due to plant-based origin from natural resources such as vegetable oils and starches.

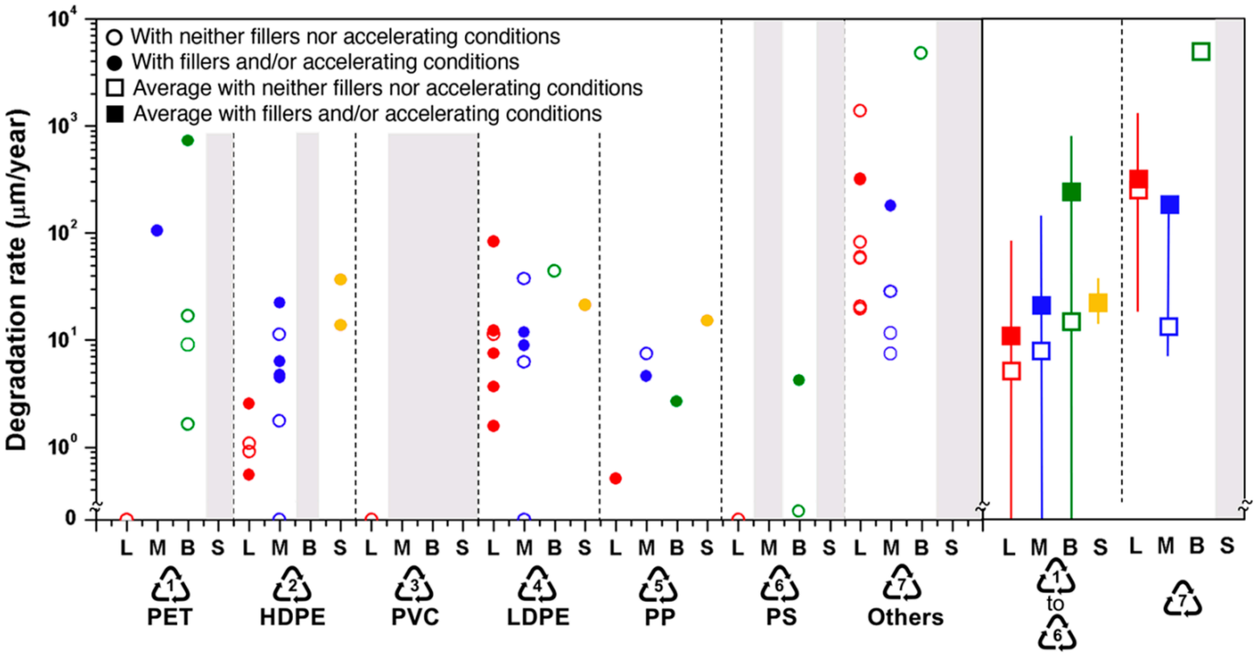


***Fig. 1.3.*** *Specific surface degradation rates for various plastics, in µm year$^{-1}$ . Vertical columns represent different environmental conditions (L, landfill/compost/soil; M, marine; B, biological; S, sunlight) and plastics types (represented by their resin identification codes). Plastics type 7, "others", corresponds to various nominally biodegradable plastics. The range and average value for plastics types 1−6 are shown on the right as lines and squares, respectively, as well as for biodegradable "others". Data points representing degradation rates that were unmeasurably slow are shown on the x-axis. Gray columns represent combinations for which no data were found.* (source: Chamas et al., ACS Sustainable Chem. Eng. 2020)

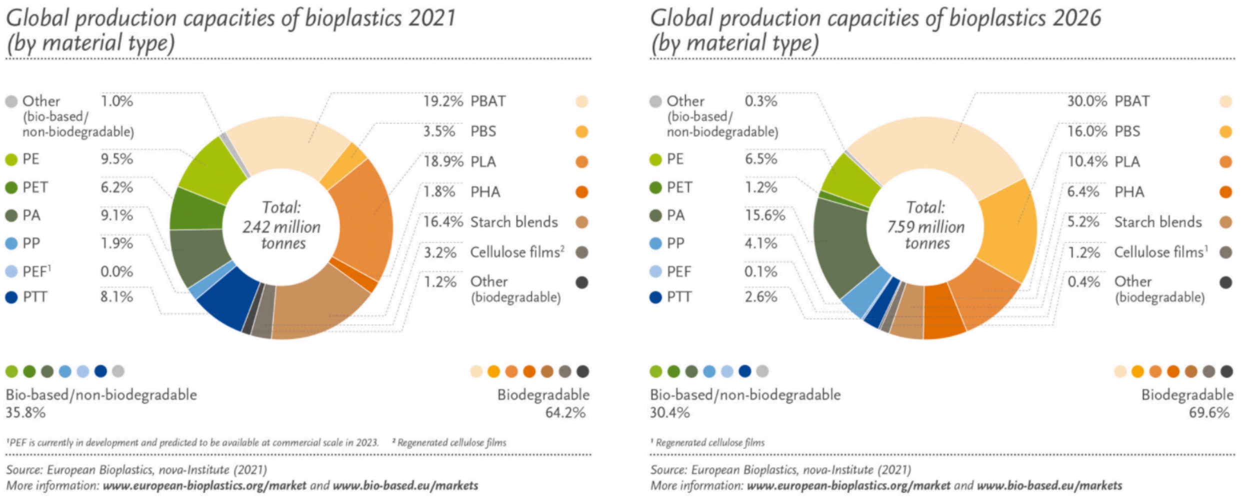


***Fig. 1.4****. Bioplastic production in 2021 and its tendency until 2026 (source: European Bioplastics, nova-Institute (2021))*

Nevertheless, at the moment, it is obvious that the time required for the degradation of annually produced volumes of plastics to their complete disappearance from the environment is a multiple of the time spent on the production and use of plastic. This state of affairs naturally contributes to the accumulation, if not of the entire produced volume, then of some part of it. For example, Eriksen et al., (2014) estimated the amount of plastic in the world's oceans at 250,000 tons. However, this figure can be much higher since such physical property of plastics as density is an important factor for its detection - denser materials (***Table 1.1***) than seawater, can sink to the bottom and become difficult or impossible to detect, and even more so to take into account in calculations. Also, it is extremely difficult to estimate the amount of plastic that ends up in landfills. Nevertheless, it is known that about 79% of the plastic produced worldwide, ends up as garbage, and only about 10% on average is recycled (cseindia.org). Due to economic inexpediency and impossibility of some types of plastics (bioplastics, composite plastic, plastic-coated wrapping paper and polycarbonate) to be recycled for technical reasons due to their properties (slrecyclingltd.co.uk).

Once in the environment, plastics begin to degrade, but the mechanical process prevails over the biological process. There is a damage and destruction of initially large objects to smaller ones, which are commonly called a secondary source of plastic pollution. As a rule, it poses the greatest danger to the environment and its inhabitants, since gradual fragmentation under the influence of the environment can reach the size of so-called micro and nanoplastics. The particles of microplastics are from 5 to 0.1 mm, and from 0.1 mm and below in case of nanoplastics, according to the most common classification (Hidalgo-Ruz et al., 2012). In this connection, almost all (or possibly all) environmental objects are to some extent bitten by both plastic and micro-nanoplastic: in the seawater and marine organisms (Alonzo et al., 2021); in the surface waters (Han et al., 2020; Fischer et al., 2016) including city canals, water treatment plants and marine sediments (Leslie et al., 2017); in the estuarine species (Pequeno et al., 2021); in the coastal shallow sediments (Alomar et al., 2016); in the mountain glaciers (Cabrera et al., 2020); in the show cave sediments (Balestra and Bellopede, 2022); in a freshwater caddisfly (Ehlers et al., 2019); in the water reservoirs (Zhang et al., 2017); in bottled and tap water (Kirstein et al., 2021; WHO, 2019; Mason et al., 2018), wine (Prata et al., 2020) and food (Li et al., 2015; Karbalaei et al., 2020).

***Table 1.1**. Density of the most prevalent microplastics (source: Crawford and Quinn, Microplastic Pollutants, 2016)*

| **Substance** | **Abbreviation** | **Density (g/cm$^3$)** |
|---|---|---|
| Seawater | SW | 1.025 |
| Polystirene (expanded foam) | EPS | 0.01-0.05 |
| Polystirene (extruded foam) | XPS | 0.03-0.05 |
| Polychloroprene (neoprene) (foamed) | CR | 0.11-0.56 |
| Low-density polyethylene | LDPE | 0.92-0.94 |
| Linear low-density polyethylene | LLDPE | 0.92-0.95 |
| High-density polyethylene | HDPE | 0.94-0.97 |
| Polypropylene | PP | 0.88-1.23 |
| Acrylonitrile butadiene styrene | ABS | 1.03-1.21 |
| Polyamide (nylon 6) | PA | 1.12-1.14 |
| Polymethyl methacrylate | PMA | 1.10-1.25 |
| Polychloroprene (neoprene) (solid) | CR | 1.20-1.24 |
| Polyamide (nylon 6,6) | PA | 1.13-1.38 |
| Polystyrene (solid) | PS | 1.04-1.50 |
| Polycarbonate | PC | 1.15-1.52 |
| Polyethylene terephtalate | PET | 1.30-1.50 |
| Polyvinyl chloride | PVC | 1.15-1.70 |
| Polytetrafluoroethylene | PTFE | 2.10-2.30 |

## PLASTIC IN THE MARINE ENVIRONMENT

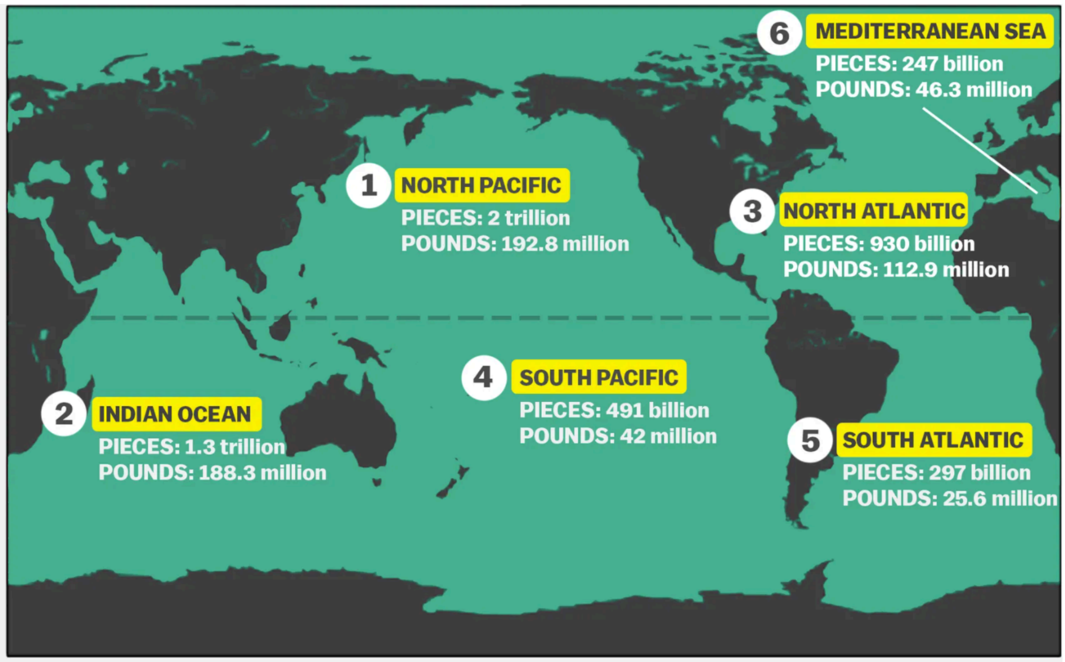
In the minds of human beings, the beach and the sea is a perfect tandem that is always perceived as paradise on Earth. Thanks to the increasingly widespread plastics, many beaches in the world have lost this status and are no longer considered as places of attraction and enjoyment, or, at least, not at the same level, or not always. Given the existence and characteristics of the water cycle in nature, almost any pollution reaches surface waters and other natural objects to one degree or another. The most obvious and massive sources of sea water pollution at the moment are:

- treatment plants that cannot cope with certain types and sizes of plastics and micro / nano plastics such as ear sticks (due to their low density), fibers from clothes and textiles after washing, synthetic nano particles from cosmetic products, etc.;

- rivers, into which the water enters from the treatment plants, as well as other waters such as household and rainwater, which have not been treated in treatment facilities and, accordingly, contain a greater amount of pollutants of various kinds, including plastic;

- ships/boats that surf the rivers and seas for commercial or trade purposes, and from the sides of which paint can chip off, mooring and fishing nets can be worn out, as well as various objects can fall out or be thrown out.

In case of plastics larger than 5 mm, the pollution problem is most noticeable. Starting from the most obvious places where plastic waste accumulates, the so-called "plastic islands" in the Pacific and Mediterranean seawaters (***Fig.1.5*** ) and the most polluted beaches such as Hawaii (Kamilo Beach in ***Fig. 1.6***) and the beaches of Asia, since 81% of all ocean plastic in the world emanates from the countries of the Asian region (Meijer et al., 2021).



NOTE: individual ocean estimates were converted from metric tons, and deviate slightly from overall estimates
SOURCE: "Plastic Pollution in the World's Oceans" (2014; Eriksen, Lebreton, et al.)

*Fig. 1.5. Plastic distribution in the world ocean (source: Vox.com)*

*Fig. 1.6. “Plastic beach” of the Island of Hawaii (Kamilo Beach) (source: nist.gov, M. Lamson/Hawaii Wildlife Fund)*

Less obvious places where plastic can be found are living organisms, the water column, and the seabed and coastal sediments. And even less obvious is the distribution and quantity in the case of poorly or completely invisible micro and nanoplastics. Such dimensions and appearance are easily perceived by living organisms as food, which is both a danger to marine life and to a person who receives water and some food from sea and river reservoirs, and therefore the consumption of micro / nanoplastic by a person can vary on average from 39,000 to 52,000 particles per year (Cox et al., 2019). At the moment, many scientists are trying to determine the impact of micro and nano plastics on the state of living organisms and especially on human health. If the physical impact of some particles is obvious, for example, broken pieces of hard microplastic can damage the intestinal tract up to death or accumulate in certain parts of the body depending on their size (***Fig. 1.7***), then the consequences of such accumulation and chemical effects on the body are still not obvious so research is ongoing. However, many scientists claim the potential provocation of such effects on the body as: toxicity through oxidative stress, inflammatory lesions, increased uptake or translocation, metabolic disturbances, neurotoxicity, and increased cancer risk (Rahman et al., 2021). Therefore, it is important to monitor pollution in various environmental objects, their presence and degree.

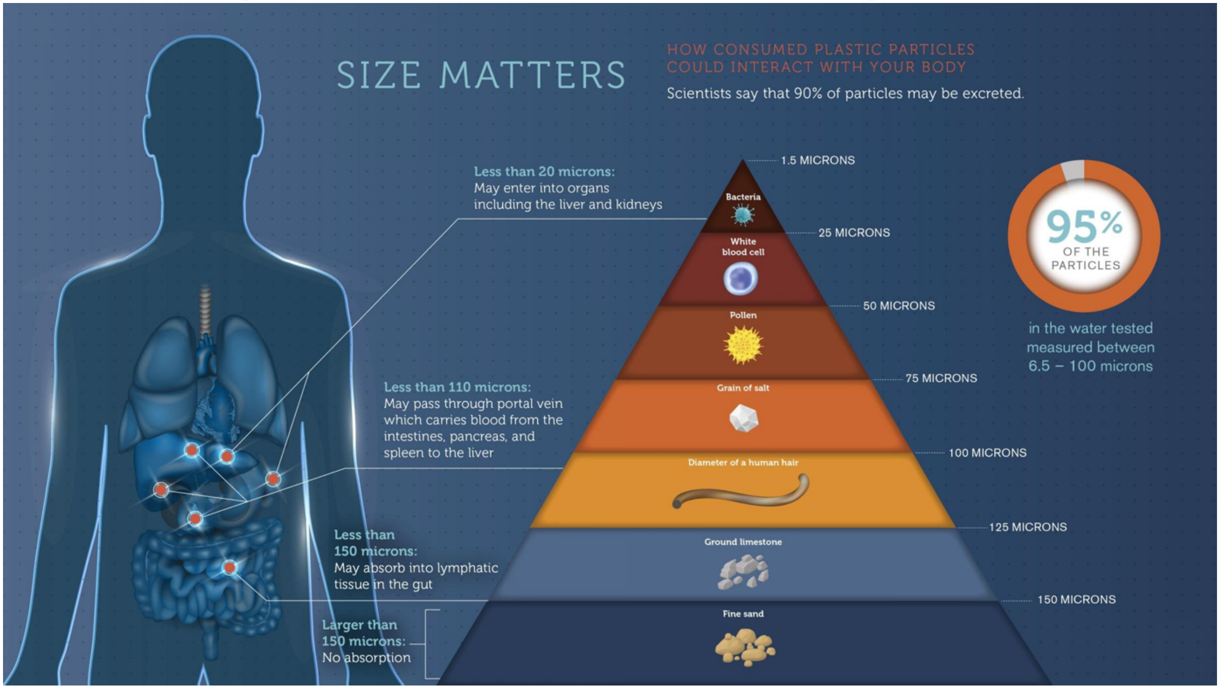


*Fig. ... Micro and nano particles interactions within human organism (source: ORB Media)*

# 2. MICROPLASTIC IDENTIFICATION AND QUANTIFICATION METHODS: CRITICAL REVIEW

As part of the study of the environment plastic pollution issue, scientists conduct research based on monitoring of the state of flora and fauna, namely living organisms, water bodies (marine and freshwater), soil and sandy coastlines (beaches) around the world, in order to determine the extent of problems and its quantitative assessment in various parts of the planet. This is necessary both for the current understanding of the scale of the problem and the study of its consequences (for the entire food chain), as well as for the subsequent development of a standard procedure for detection (i.e. separation from the environment of animate and inanimate nature in which microplastics are located), identification and their calculation. The standardization of procedures will allow setting of the lower and upper threshold limits for the content of microplastics, for example, in drinking water, which is subsequently used in the production of a huge number of products, in addition to its direct consumption. Thus it allows manufacturers, treatment plants and regulatory authorities to focus on certain indicators, which is currently unrealistic. Despite the variety of methods and approaches at each stage, by now the following fundamental order of manipulation with sand material, and the microplastics, potentially contained in it, has been established: sampling, separation / purification, identification and counting of microplastics.

## SAMPLING

The strategies and methods of material collection vary depending on which medium should be analyzed. For sand and water environments, strategies are globally divided into 2 categories, which depend on the size of the plastic contained in the sample. With the size of plastic visible to the naked eye and easily recognizable among other objects, a selective technique is often used to take only plastic material and separate it from sand directly on the spot. The second category includes two methods of sampling material containing small microplastic sizes, namely bulk and volume-reduced sampling methods, in which sand material is collected without or with reduction in the volume of material at the site of the object under study, respectively (Hidalgo-Ruz et al., 2012; Xiang et al., 2022). For the study of biota, as a rule, individual specimens are selected or purchased for subsequent research in the laboratory, but also groups using meshes of different sizes (Leslie et al., 2017; Liu et al., 2021).

When the methodology is determined, it is necessary to choose with what and how, as well as where it will be carried out. And here techniques and approaches are much more diverse in many ways, because researchers pursue different goals, in addition to the main one - determining the presence of microplastics, they can also compare in which zone and /or at what depth the largest amount of microplastics is located. In the case of beach samples, as a rule, preliminary zoning of the analyzed place is carried out, and it can extend both to the widest possible area of the beach and to individual strips of different distances from the sea (Hidalgo-Ruz et al., 2012).

## SEPARATION

If at the first stage of sampling bulk and volume-reduced methods were chosen, then the next stage is the separation of the prepared material and the extraction of microplastics from it. The separation procedure also differs depending on the type of material being analyzed. Sand material is usually subjected to sieving and then to densimetric separation (Hidalgo-Ruz et al., 2012; Liu et al., 2021). But sometimes there are  the tests using an electrostatic separator being performed as well (Enders et al., 2020; Felsing et al., 2017). Since all sampled material, especially in the case of the bulk sampling method, cannot be tested in full (especially if large volumes are involved), it must be subjected to a volume reduction procedure and subsequent analysis of only a small part. Usually it is about from 10 to 60 grams on average of sampling (Vermeiren et al., 2020; Enders et al., 2020; Jahan et al., 2019; Balestra and Bellopede, 2022; Leslie et al., 2017).

### ➢ *Granulometric sepration*

Preliminary granulometric separation of material using meshes of different sizes (from 0.038 to 4.75 mm) is often used to distinguish and separate the material and the microplastics contained in it into the classes and/or to clean samples from larger material (Hidalgo-Ruz et al., 2012). But the reverse side of this process may be the loss of microplastics and, accordingly, the underestimation of degree of pollution, and this is probably why the use of granulometric separation is not so common in works after 2012. Thus, this step can be applied or skipped depending on the specific goals of the work and the type and size of the material being studied. For example, if it is visually noticeable that the sand is too small and there are no too large particles in it, i.e. more than 3-5 mm (Liu et al., 2021), or if such large material is present, it can be selectively removed with tweezers (NOAA, 2015).

### ➢ *Electrostatic separation*

This is a widely used technology in the separation of waste into fractions, especially WEEE (Waste Electrical and Electronic Equipment) and various wires and cables. Separation occurs through the use of electric forces that are able to separate the conductive material (that conduct electricity) from the non-conductive material, which is polymers.

Using this technology to separate microplastics from the rest of the sample, it is possible to use a larger amount of material, on average 100-150 grams, and thus simultaneously increase the concentration of microplastics in the reduced volume of the sample. Such an approach can help both for laboratory analysis for microplastic contamination and for the direct cleaning of large volumes of beach sand from plastic and microplastics. Studies conducted on this topic show different results, but in general they are quite optimistic. For example, the study by *Enders et al. (2020)* have demonstrated recovery efficiencies ranging from 45 to 100% depending on the size of the microplastics contained (from 20 micrometers to over 2 mm) in the sand i.e. as the size decreases, the efficiency naturally decreases. In addition, a decrease in efficiency has been seen in the extraction of microplastics from commercial sand due to the higher content of fine calcite particles (<50 µm) and also from soil samples (for the same reason). The high recovery efficiency (99%) is also confirmed by *Felshing et al., 2017*. However, in this study were analyzed the different types and sizes of plastic prepared in the laboratory and mixed with different types of sand and sediment. Which confirms the conclusion of *Hidalgo-Ruz et al.,* *2012* that good results can only be obtained with an artificial sample, but not with a real one taken from the environment and containing organic material.

Despite the possible losses, one of the additional positive points in the use of an electrostatic separator may be the uselessness of the subsequent use of densimetric separation if microplastics are analyzed in the range from 0.450 to 4 mm (Enders et al., 2020). Accordingly, despite the reduction in volume, electrostatic separation is currently not able to completely separate microplastics from sand so effectively that it is possible in order to analyze potential microplastics smaller than 0.450mm without applying densimetric separation, and, as a next step to separate the remaining denser inorganic material from microplastics.

- ***Densimetric separation***

Densimetric separation is the most widely used method and has proven to be effective, and it is usually used alone (Enders et al., 2020). It is a solution that is able to carry and maintain material with the lowest density on its surface due to its increased density. Thus, the vast majority of types of microplastics with a lower density in the range of 0.8–1.4 g/cm$^3$ (Xiang et al., 2022) are able to float to the surface of a denser solution (1.2–1.62 g/cm3) after mechanical mixing of the solution with sand material, which in turn, it settles because it has a higher density, 2.7 g/cm$^3$ on average (Liu et al., 2021). However, there is still no unanimity among the studies on the basis of this type of separation, namely which particular solution is preferred among numerous options, each of which has its own pros and cons. This happens because of some mortar bases, such as NaCl, having a maximum of 1.2 g/cm3, is not able to hold denser plastics on the surface, but which is the most environmentally friendly. However, it is clear that despite the high popularity of this solution among scientific papers, its use may lead to an underestimation of the amount of microplastics with higher density, such as polyvinyl chloride (1.4 g/cm$^3$). Along with this solution, the use of $ZnCl_2$ with a density of 1.5 g/cm3 is also widely used, which is able to make denser plastics float. Other solutions are also used (NOAA, 2015; Lastovina et al., 2020): lithium metatungstate (1.62 g/cm$^3$), CaCl2 (1.30–1.35), NaI (1.57), ZnBr2 (1.7), NaBr (1.37), sodium lauryl sulfate (SLS), sodium polytungstate (SPT), etc.

## ORGANIC MATTER REMOVAL

The last step in the preparatory process before identification is organic matter removal or digestion, the purpose of which is to remove organic impurities by dissolving them. Various solvents are used in different concentrations, depending on the origin of the material and, accordingly, a higher or lower organic content. Thus, sand and sediment samples require a concentration of at least 30%, the most commonly used is the hydrogen peroxide (H2O2) solution. Along with this, Fe(II) solution (0.05 M) is also often used. Moreover, some researchers suggest using both of these solutions sequentially to better eliminate organics (Liu et al., 2021), since the use of only H2O2 (30%) may not be enough and subsequently lead to an overestimation of microplastic due to its similarity to some organic materials, as well as a possible underestimation if the remaining organic material overlaps the microplastics.

For the most part, it is observed that the application of these (and other) solutions occurs on already filtered and dried material - the so-called dry digestion. But there are also studies that use the “wet” method; they add a solution at the stage of densimetric separation, and also use both methods sequentially (Kang et al., 2020).

## IDENTIFICATION

### ➢ *Identification with microscope under UV light*

The optical microscope is widely used and applied in the identification of MPs and refers to physical or visual identification. From all the tools currently available, this one is the most accessible and relatively easy to use, allowing to identify the color, shape and size of a plastic or other particle under ordinary lamp light in order to distinguish them from each other based on certain criteria. Fluorescence microscopy is considered a slightly more advanced method, since it allows additional detection of transparent particles, which in most cases and in a regenerating amount are transparent fibers due to their entry into various natural objects and subsequent natural influences (for example, abrasion, weathering, chemical and physical destruction). Indeed, in many scientific works, the greatest presence of precisely fibers, and often transparent ones, has been revealed. Therefore, the use of autofluorescence of identified particles is a necessary step and greatly simplifies the task of their detection. Despite the fact that fluorescence is characteristic not only of plastic but also of other objects, as well as various anthropogenic impurities and dyes used by humans and, moreover, some plastic may not fluoresce at all or do so under a certain filters, that is, not universal for each type and respectively revealing one type but not another. For this purpose, a properly selected UV light lamp can be a more cost-effective alternative in comparison to a fluorescence microscope (Balestra e Bellopede, 2022).

Despite the fact that at the moment there is no generally accepted methodology either in general or for identification of microplastics in particular, nevertheless, the main criteria used in most scientific works or based on them in one way or another are the characteristics proposed by *Norén et al. (2007*), namely:
- lack of organic and cellular structure in the studied objects;
- in case of fibers, their width must be the same along the entire length, not tapering towards the ends and have 3D bends, otherwise their straightness may indicate the organic origin of the object;
- the color should be clear and homogeneous in the case of colored objects, while ghostly and whitish ones should be studied with more attention using additional magnification and a fluorescent microscope.

Surprisingly, the strict/classic criteria proposed by *Norén et al* back in 2007, which guide most scientific papers on the topic of microplastics, do not always themselves meet these criteria, as evidenced by photographs of examples of microplastics in these works (even if they declare adherence and compliance with the criteria), especially this discrepancy concerns fibers and the so-called impact PP (Song et al, 2015). So, *Norén et al* in his 2007 work, under the rules (mentioned above) imposted by himself, published as an example a photograph of a blue formation fiber (***Fig. 2.1***), which hardly meets his criterion of the same thickness over the entire surface. This illustrates the subjectively of these rules that can be perceived and interpreted differently even for their author, and even more so for other people. This accordingly provokes a large number of errors and as a result of both overestimation and underestimation of the studied environmental object.

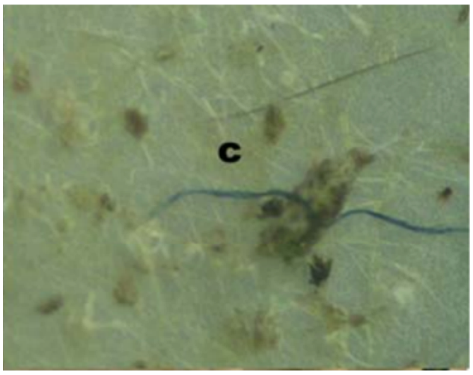


***Fig. 2.1***. *Example of a non-tapering synthetic fiber by Norén et al., 2007*

This thesis is confirmed in the MP identification methodology released by the *Marine & Environmental Research Institute (USA, 2015)* in order to unify the system for determining and counting microplastics, in which the authors, based on the classical criteria of *Norén et al (2007)*, nevertheless emphasize that they can vary because microplastic itself and each of its particles are very diverse, and under each of the criteria they give visual examples of variations in classic characteristics and how one or another sample can be discarded due to non-compliance with one or another criterion, while being a microplastic. But despite this, it is still advised to adhere to conservative criteria and, in case of doubt, do not take into account this or that object.

Then *Hidalgo-Ruz et al. 2012* in their work (one of the most cited scientific articles on the topic of microplastic pollution on which many more recent works are based) analyzed more than 60 other scientific papers and, among other things, and they came to the conclusion that visual identification is necessary in accordance with the above criteria, but setting a lower threshold for possible identification with this method is 1 mm, below which the error level progressively increases and, therefore, this method cannot be reliable. Also, *Hidalgo-Ruz et al.* devoted most of her article to the analysis of MF susceptibility to fragmentation, weathering, various types of degradation depending on the time spent by microplastics in a particular area and depending on other factors, as well as the consequences of these impacts. Thus, within the framework of one study, the inconsistency can be noted since the conservative criteria for the appearance of the MPs hardly correlate with various processes that change their appearance. And, accordingly, may partially or completely cease to correspond to them, but continue to identify the modified MPs using classical criteria without taking into account some external influences. Thus, a few classical criteria existing nowadays does not correspond to the diversity of both the microplastics itself and its modifications.

The ambiguity of the findings in *Hidalgo-Ruz et al. (2012)* is also confirmed by the experiment performed by *Shim et al. (2016),* where was partially recreated the typical natural conditions to which coastal plastics are exposed, but in laboratory: the fragmented microplastics (PP) were exposed to ultraviolet light for 6 months and mechanical abrasion causing by sand, and a roller mixer for two months. As a result, firstly, even preliminary densimetric separation did not completely separate microplastics from sand, which in its shape, size and texture was so similar to microplastics that neither micro-FT-IR (Fourier-transform infrared spectroscopy) nor, moreover, identification with using a microscope, they could not recognize most of the PP microplastics (98.7% having a size <300 μm), but only a few dozen particles, despite numerous and many hours of attempts with the ATR

(Attenuated Total Reflectance Probe). However, previously unrecognized microplastic particles were successfully identified by staining with Nile Red dye under green fluorescence, and then their synthetic composition was further confirmed using FT-IR.

Therefore, in addition to the complex, exhausting and time-consuming visual identification itself (and the problems associated with this, such as reducing the vigilance of the researcher), the task of correct identification of MPs is complicated by various weather conditions and natural phenomena that significantly and indefinitely affect certain types of MPs, especially in sandy deposits and sea / fresh water, which leads to additional ambiguity and inability to follow more or less well-established criteria, especially classical ones. Thus, more and more scientists, who noticing this problem, slightly deviate from the classical criteria by adding new ones (Sun et al. 2019; Nor and Obbard, 2014) and / or correcting the old ones, and also propose to define a particle as plastic only if it simultaneously meets at least two criteria. (Horton et al, 2016; Windsor et al, 2019), which in turn can also lead to additional quantitative underestimation. But even this is often not enough, and therefore, in order to reduce the error and dispel numerous doubts, additional more advanced, but more expensive and time-consuming methods of MPs identifying are used, which can not only confirm or refute whether the object under study is plastic, but also determine its chemical composition (although this is not always possible, as it depends on specific databases and their content), which in turn can help in the analysis of the origin and, accordingly, tracking the source of pollution. Some researchers even skipped preliminary identification using a microscope (Cincinelli et al, 2017) and immediately switched to recognition using analytical methods due to the unsatisfactory results of colleagues or their own past work using a microscope (Song at al, 2015; Prata et al., 2020), which confirms the trend towards the forced use of more precise and progressive techniques. So, *Cowger at al (2019)*, after analyzing of many scientific papers on microplastic pollution and its identification, asked a fundamental question: how reliable can these works be? Since 14 of them (most of those analyzed by him) did not have verification through analytical methods, and have based their plastic count results only on identification through visual analytics, which, in his opinion, cannot be a reliable result since the error level may be too high. In this regard, it is necessary to make sure at least (in the presence of a large number of samples) by statistical (Hanke et al., 2013) or sampling method (Jiang C. et al, 2018; Cheang CC et al, 2018), selecting individual samples (for example, the most frequently repeated and / or vice versa rarely occurring characteristic elements) and additionally identifying them using analytical techniques. But these progressive methods also have their drawbacks and may not always give accurate results (Irfan, Tahira & Khalid et al, 2020; Turner et al, 2019). A comparison of the various applied techniques, their pros, cons and limits was analyzed by *Mariano et al, 2021* and *Woo et al., 2021*.

Another side of the problem with the current approach is that microscopic studies of particles smaller than 0.5 mm enclose an error of the order of 20 percent and increase to 70 percent, followed by a decrease in size (Eriksen et al. 2013a; Hidalgo-Ruz et al. 2012). However, even expert researchers cannot accurately determine, following all the criteria of a conservative approach, whether the examined particle refers to plastic or something very similar to it, i.e. so-called plastic-like particles. In this regard, many scientific works put the upper limit for identification under the microscope of at least 0.5 mm or even in 1mm, in case the research is limited to visual identification and its results cannot be confirmed analytically i.e. through advanced study of the chemical composition of the particles (FT-IR, Raman). Similarly, the *European Commission*, , in its proposal for methodology *(2013)* for monitoring pollution from microplastics, by setting a lower identification limit of 0.1 mm when using a microscope, still recommends supporting this study with spectroscopic technology and to subject at least 10% of the subjects studied particles to further verification (Song et al., 2015; Lavers et al., 2016; Xu et al., 2019; Bertolidi et al., 2021).

Therefore, it is the main problem because according to many scientific studies there is a trend that the number of microplastics increases with a decrease in its size (Song et al, 2015; Hengstmann et al., 2019) and, consequently, the main range in where more particles of microplastics are found is 0.1-0.250 mm (Bertolidi et al., 2021; Limbago JS et al. 2021; J. Li et al 2015; Feng et al, 2021) or >0.250mm (for do not set a lower bound that is hardly identifiable) i.e. a category with the likelihood of a large error when using visual identification only. This fact poses the main problem because according to many scientific studies, the essential range in which the majority of microplastic particles are found is 0.1-0.250 mm. Moreover, using conservative criteria in the identification of microplastics, a significant underestimation of the number of particles by about 1.5 times compared to the FT-IR identification method (Song et al. 2015), since many particles are discarded, which cause doubts to the expert, and the less the analyst is experienced, the more this underestimation increases, which leads to a more global underestimation of the state of a particular natural object, the correct evaluation of which is the original purpose. It turns out that in the same sample (depending on its type), paradoxically, the amount of plastic can be both underestimated and overestimated on the basis of some incorrect conservative criteria applied: what is not taken as plastic – remains (especially what regards to fibers), at the same time what is real plastic is being rejected (in practice it happens with fragments). The first classical criteria *Norén et al (2007)* in his research advises not to consider transparent fibers due to their high similarity to the natural fibers, thus it demonstrates the unreliability of the visual method, as well as knowingly tolerating a very significant error because the results of some part of the research demonstrate the great advantage of transparent synthetic fibers among the entire volume of the sample under study, depending on the object under study. (Takarina et al, 2022; Feng et al, 2021, Balestra and Bellopede, 2022; Wu et al , 2018; Jiang C. et al, 2018; J. Li et al 2015; Feng, S. et al, 2021; Pradit et al, 2020; Hidalgo-Ruz et al. 2012). It follows that human error in this kind of study plays a vital role, while the scientific world is trying to reduce it (or eliminate it) in all areas.

Thus, the experience of scientists expressed by them in numerous scientific publications (Song et al. 2015; Loder and Gerdts 2015; Kroon et al., 2018; Hidalgo-Ruz et al. 2012, Xiang et al, 2022; Mariano et al, 2021; Dekiff et al. 2014; Wang at al, 2017; Campanale et al 2019; Bertolidi et al, 2021; Eriksen et al., 2013; Strand, Jakob & Tairova et al, 2015; Pequeno et al, 2021; Limbago et al, 2021; J. Li et al 2015; Jiang et al, 2018; Stand et al, 2015; Fischer et al, 2016; Blair et al, 2019) it can be concluded that this method alone, without help of other methods, cannot be optimal and reliable for identifying MPs less than 1mm, and even less than 0.5mm, especially in soil and sand samples where visual identification is further complicated by the presence of a large number of organics and minerals (Ehlers, Sonja & Manz, W et al, 2019; Thomas, D et al, 2020; Nguyen et al, 2019). Visual identification itself requires serious preliminary preparation on the side of a person analyzing the presence of microplastics. But even passing a preliminary training to detect the MPs and minimizing the subjectivity of the results does not always eliminate a significant error, about 40%, in the correct identification of the MPs, despite the fact that during the training the results of determining and calculating the test sample of the *Fischer et al (2016)* group of scientists varied only 10% apart.

### ➢ *Identification using Nile Red dye*

Due to the fact that about 70% of detectable microplastics are transparent or white, which difficult to identify under a microscope, even with UV light. There was a need for an alternative method of identification that can stain poorly visible microplastic particles. One of such methods was the organic fluorophore NILE RED (or the heterotetracyclic compound 9-(Diethylamino)-5H-benzo[a]phenoxazin-5-one ***(Fig. 2.2.)*** according to the Preferred IUPAC name), which is a lipophilic-type salvatochromic dye originally used in microbiological and biomedical purposes. Nile Red formula $C_{20}H_{18}N_2O_2$ was designed for fluorescent detection of various cell molecules (lipids, lysosomes, etc.) as well as labeling of nanoparticles, etc. Relatively recently, this technology for the use of lipophilic dyes was proposed by the North American scientist *A. Andrady (2010)* for the identification of microplastics and subsequently widely used by his colleagues around the world. In the case of microplastic pollution studies, this composition binds to the surface of a plastic polymer due to the attractive forces of van der Waals, after which it begins to glow with different intensity and a certain color under light rays from a certain part of the spectrum, depending on the polarity of the medium in which NR is dissolved, as well as on the hydrophobicity of the studied synthetic material itself.

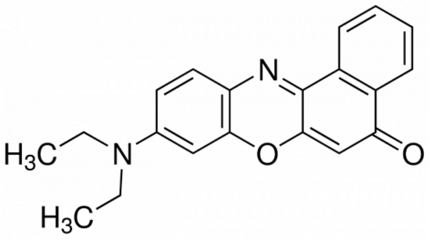


***Fig. 2.2****. Chemical structure of the dye Nile Red*

It has been observed that the analyzed works on the topic of microplastic indication using NR dye often contradict each other, sometimes giving different staining results for the same specific types of plastic (usually prepared in the laboratory, but also obtained from natural objects). For example, according to an experiment by *Shim et al., 2016* NR were able to dye PP and EPS (among others) but not PVC, polyester (PES), PA and PET. While *Ivanova et al. (2020)* and *Tamminga et al. (2017)* were able to color PVC.

A study by *Veitur et al., 2019* summarized the unsatisfactory result of dyeing polyester, which is currently the dominant contaminant. This correlates with other results regarding PVC staining.This is because although NR staining technology is generally considered to be a fairly easy-to-use and economical method of detection, nevertheless, its ins and outs are not yet well understood (Liu et al., 2021). In this connection, there has been a tendency to study it more closely under various conditions (although at the moment the share of studies related to fluorescent identification is only 11% of all studies involved in the identification of microplastics (Liu et al., 2021). Since the result of staining and, accordingly, the appearance of a stronger or weaker fluorescent signal depend on many factors, such as following: the color of the filter on a fluorescent microscope and the excitation wavelength, the type of solution and the concentration of NR in it, the adsorption time at room temperature (for the reaction) and the exposure temperature at oven drying, as well as the correct pre-treatment of the sample with digestion and other less understood factors, such as the effect of solvent pH on the staining process (Sturm et al., 2020). The ***Table 2.1*** shows some of the staining techniques with NR and the variability in the study of some of the above parameters that affect the final staining result and identification to a greater or lesser extent. The fundamental factors influencing NR staining are described below (***a, b, c, d***).

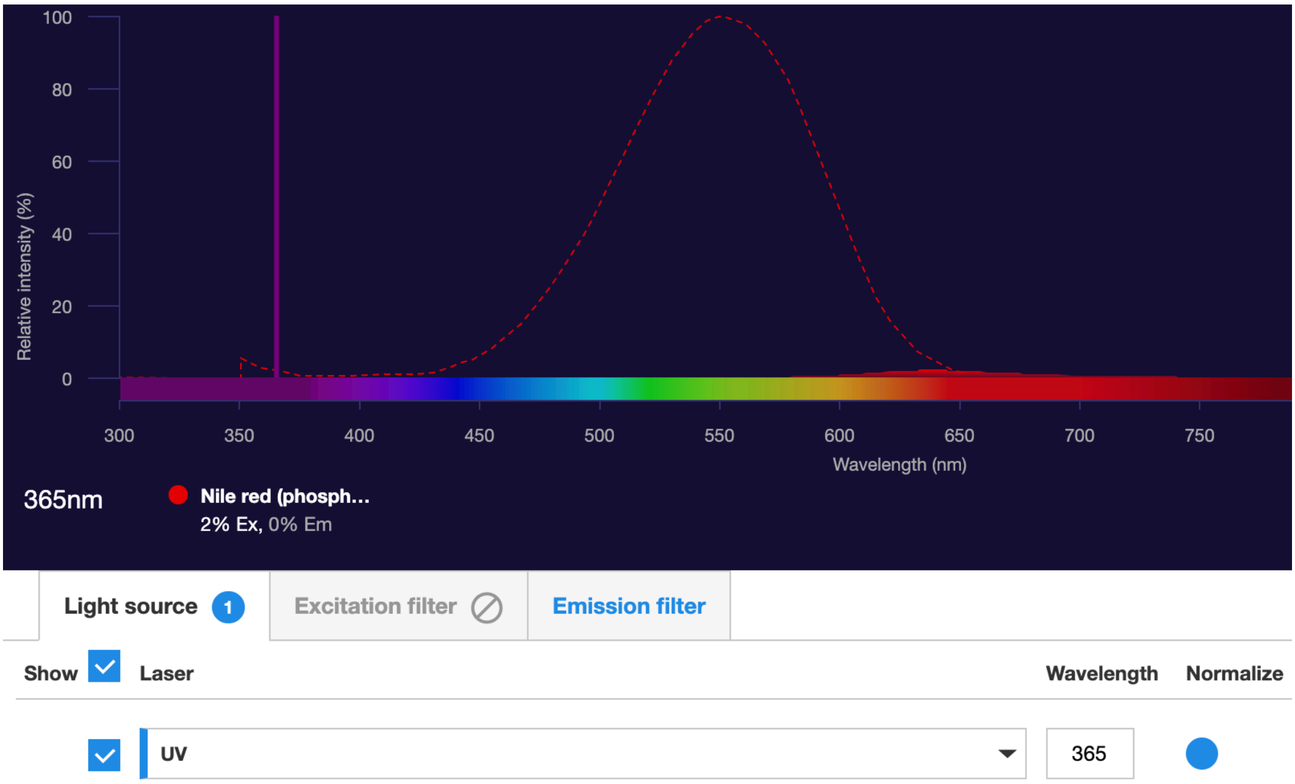
***Table 2.1.*** *Comparison of techniques using Nile Red dye*

| Equipment | Pre treatment method | Filter | Concentration NR/Solvent (µg/ mL) | Solvent | Author |
|---|---|---|---|---|---|
| UV-light (Omnilux UV ES 365 nm) using a UV-microscope (PCE-MM 200 UV 365 nm); Scanning Electronic Microscope | hydrochloric acid for 48 h at room temperature and additional hot digested at 70 C for 1 h | glass microfiber filters 691 VWR International (particle retention 1.6 µm) **AND** qualitative filter 413, VWR International (particle retention 5–13 µm) | 1000 | acetone | Fischer et al., 2016 |
| Fluorescence Microscopy; blue light source (460 – 470 nm); Orange high-pass filter (Filter G350)<br>Dark Amber by Rosco Laboratories Inc | 10ml; 1mol/L NaOH | stainless-steel filters (custommade by Inoxia Ltd., Great Britain) aperture of 27µm | 1000 | acetone | Veitur, HS Orka and Norðurorka by ReSource International ehf., 2019 |
| automated filter-scanning rig; blue light and orange filter; infrared (IR) microscopy | - | Whatman cellulose nitrate membrane filters; PTFE syring filters; Anopore filter | from 1 to 1000 | acetone | Maes et al., 2016 |
| fluorescent microscope with 3 different wavelength filters (blue, green-yellow, orange-red) | ($H_2O_2$) solution (35%) for sediment semples; 0.05 M Fe(II) solution and 35% $H_2O_2$ for Floating microplastics | polycarbonate (PC) filter | 0.5 and 5 | acetone and n-hexane | Shim et al., 2016 |
| fluorescence microscopy (green and red filters) and image analysis software (automated ImageJ quantification); Micro-Raman spectroscopy | 30% $H_2O_2$ | PC track-etched filter membranes (PCTE, 25 mm diameter, 10 µm pore size, Whatman) | 1 | metanol | Enri-Cassola et al., 2017 |
| LED light source and filter (410–490 nm) and color filter (532 nm); Digital camera | - | - | 20 | ethanol and acetone | Konde et al., |
| light microscope; 455nm LED flashlight | 10% KOH | glass filter | 10 | acetone e hexane | Valine, 2019 |
| Stereoscopic Zoom Microscope and Fluorescence Microscope with 3 different wavelength filters | zinc bromide | Whatman filter paper, 55 mm dia. of 1.2 µm particle size retention | 0.5 | acetone e n-hexane | Michelaraki et al., 2020 |
| FT-IR | $H_2O_2$ (30%) and Fe(II): $H_2O_2$ + Fe; 1:1 v/v | - | 0.01 | etanol | Prata et al., 2020 |
| UV wavelength of 365 nm; stereoscopic microscope and camera; FT-IR spectroscope | 100 mL of 35% $H_2O_2$ (wet) and 2 mL of 0.05 M Fe (II) sulfate and $H_2O_2$ (dry) | track-etched polycarbonate PCTE filter, pore size of 20 µm and a diameter 47 mm (GVS, Sanford, ME, USA) | 10, 100, and 1000 | chloroform | Kang et al., 2020 |
| fluorescent microscope (red and green lasers); ImageJ analysis; Scanning electron microscopic; Raman and FT-IR spectrometer | 50 mL of KOH (10%, m/v) | PTFE membrane (0.22mm; 47 mm) | 10 | ultra-pure water and DMSO (v/v ¼ 1:1) | L. LV et al., 2019 |
| fluorescence microscope with 3 Fluorescence filters (0range, green and red) | 35% $H_2O_2$; 20 ml PBS solution (phosphate-buffered saline) and 20 ml chitinase | black filter membrane (Metricel® Black PES, pores: 0.45 µm, diameter 47 mm and 25 mm). | from 5 to 0.005 | acetone and isopropanol/acetone | Sturm et al., 2021 |
| white and green light, mFT-IR | Fenton's reagent | GFF filter | 10 | acetone and distilled water | Vermeiren et al., 2020 |
| fluorescent microscope with 2 different wavelength blue (UV: 330–385nm) and green (510–560 nm) | $H_2O_2$ (30%) | - | 10 | n-hexane | Ivanova et al., 2020 |
| UV light photobox (365 nm) vs fluorescence microscope, µ-Raman spectroscopy | $H_2O_2$ (30%) | VWR, qualitative filter paper 413, 5–13 µm particle retention | 1000 | chloroform | Hengstmann et al., 2019 |
| fotocamera under UV-light (365 nm); SAGA (System for Automated Geoscientific Analyses); green, red and blue light channel | - | filter membrane (413, VWR International, particle retention 5–13 µm) | 1000 and 10 | chloroform | Tamminga et al., 2017 |

### a) excitation wavelength and filter color

In the identification of microplastics by NR staining, fluorescence spectroscopy plays an important role, which is a type of electromagnetic spectroscopy within which the fluorescence of a sample is analyzed, and one of the main characterizing parameters is the radiation intensity and extraction/emission wavelength, measured in nanometers (nm).

On ***Fig. 2.3*** a comparison of extraction/emission parameters and relative intensity in percentage terms with different light sources or wavelength filters available for the fluorescence microscope (UV light, green and orange light) has been demonstrated. As can be seen, the most effective fluorescence spectrum is the green range (Michelaraki et al., 2020) i.e. green emission (530/27 nm) or, in a broader sense, the green-yellow range with excitation wavelength from 450 to 490 and emission wavelengths from 515 to 565 nm, which is also confirmed by other experiments that tested different filters using a fluorescent microscope (Enri – Cassola et al., 2017; Shim et al., 2016; Tamminga et al., 2017; Sturm et al., 2021). Orange is also the second most preferred option after green (Valine, 2019), followed by red, cyan and UV. It has been studied that UV light can be reliable for microplastics > 0.63 mm without using a microscope but only a photobox, but when using a fluorescence microscope (without color filters), the reliability of the method increases (Hengstmann et al., 2019). However, this method is suitable for recognizing a limited type of microplastics that are able to fluoresce at 365 nm UV extraction. Whereas at an excitation wavelength of 470 nm or more, a greater number of plastics with different chemical compositions can be seen and identified (Liu et al., 2021; Michelaraki et al., 2020).

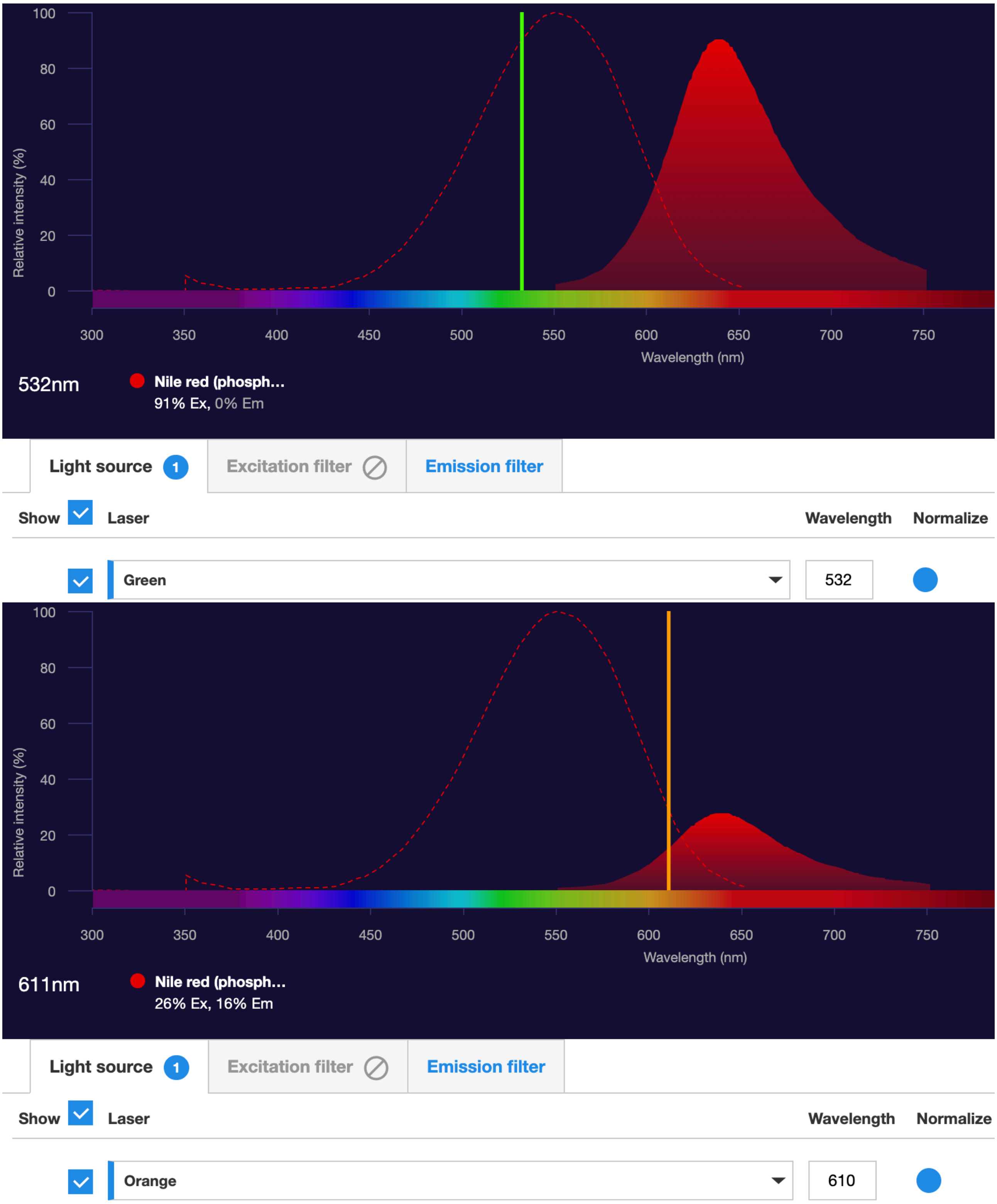


365nm

● Nile red (phosph...
2% Ex, 0% Em

Show ☑ Laser | Wavelength

☑ UV | 365



532nm

Nile red (phosph…
91% Ex, 0% Em

☑ Green — 532

611nm

Nile red (phosph…
26% Ex, 16% Em

☑ Orange — 610

***Fig***. *2.3*. *Nile Red spectra normalization to a specific laser/ wavelength filters (sourcet: Thermofisher scientific): under UV, Green and Orange light*

### b) type of solution and NR concentration in it

However, the intensity and color of the radiation is affected not only by the type of filter, but also by the type of solution that is used for staining, thus increasing or decreasing the fluorescence intensity of the identified object due to its hydrophobicity, that is, depending on the degree of polarity of the organic solution, its ability to stain the material increases or decreases (***Fig. 2.4***). For example, water, being a strong polar solvent, practically does not allow Nile Red to show its fluorescent properties, in addition, there is a tendency to shift towards higher wavelengths when using higher polarity solvents (Sturm et al., 2021). Therefore, among scientific works devoted to or affecting the topic of identification of microplastics using dyes, there are often experiments with different solutions to identify the most effective one. For example, *Tamminga et al. (2017)* after testing n-hexane, chloroform and acetone concluded that the most effective extraction solvents for Nile Red staining procedure is chloroform for most of the most commonly encountered types of plastics, while less influencing (staining) biogenic matter compared to other solutions. While other researchers (Shim et al., 2016; Michelaraki et al., 2020; Valine, 2019) have estimated the greater effectiveness of non-polar n-hexane, however, in this case, there is a problem of poor solubility of the Nile Red dye in it, so researchers often resort to the initial dissolution of Nile Red in a small amount of acetone (a few ml), and then the resulting concentrate is diluted with a solution of n-hexane to obtain the required working concentration. Nevertheless, the most common solution in research is acetone, which has an intermediate polarity. There are also successful experiments with mixing different solutions, such as acetone and ethanol (Konde et al., 2020).

In addition to the solution itself, the concentration of Nile Red in it is equally important. Some studies have found a correlation between fluorescent signal intensity and an increase in NR concentration in solution (Sturm et al., 2021; Konde et al. 2020, Maes et al., 2017). However, it has also been noted that too strong concentration can lead to saturation of spectrometer intensity and even the opposite effect (Enri-Cassola et al., 2017), as well as a natural and senseless increase in the cost of the study. On the other hand, *Prata et al., 2020* advises not to use a concentration lower than 0.01 mg mL−1, otherwise it will lead to loss of fluorescence. While the upper threshold is very different from study to study, and sometimes contradict each other. For example, *Liu et al., 2021* advise not to exceed a concentration of 20 µg/mL, while *Kang et al., 2021* noted an increase in signal intensity up to a concentration of 1000 µg/mL, as well as other researchers using this concentration (Fischer et al., 2016; Hengstmann et al., 2019).

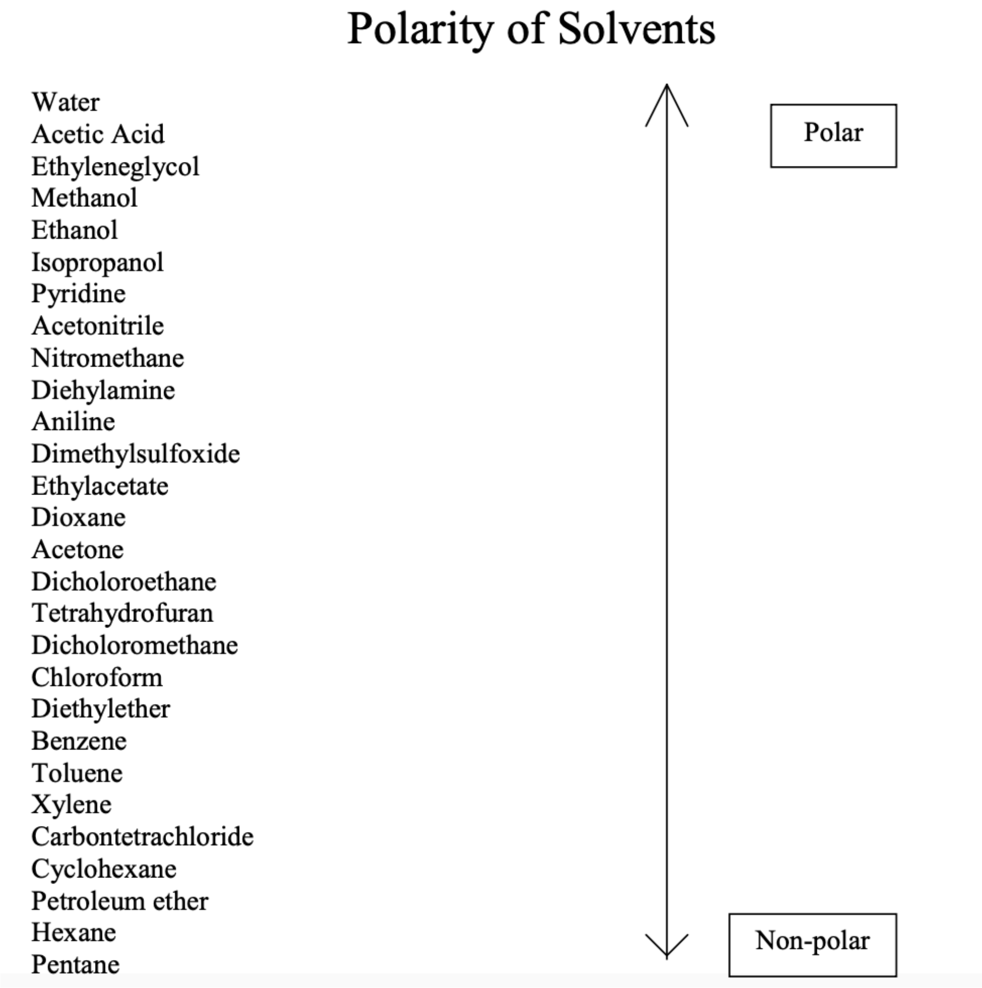


***Fig. 2.4.*** *Intensity of solvent polarity (source: chemistry.osu.edu)*

### c) staining time at room temperature and exposure temperature in oven

After applying the working concentration of the solution containing the NR dye to the test objects, it is necessary to wait for the incubation/staining time, which varies from several minutes to several hours. So *Maes et al., 2017* found that this time should not exceed 30-60 min, after which the plateau stage and gradual aggregation of the unabsorbed dye sets in. However, with a decrease in this time (less than 30 min), the intensity of acquired fluorescence decreases, which is also confirmed by *Prata et al., 2020*.

Also important is the time and temperature in the oven. Here, time only affects the drying of the filter, while temperature also affects the subsequent intensity of the fluorescent signal. It has been observed that drying from 20°C to 50°C the most effective temperature is 50°C for most plastic types. While for PVC this threshold is reached at 75°C (*Liu et al., 2020*). However, plastics have different melting points and this must be taken into account when choosing a general drying temperature.

### d) type of plastic

Despite all the above and analyzed conditions, the problem of plastic detection using this staining technology is further complicated by the internal characteristics of the plastics themselves, that is: their hydrophobicity, color, shape, as well as physical characteristics that affect, among other things, the ability of one or another plastic melt under the influence of temperature and/or digestion solvent. These features differently influence their predisposition to staining and consequently subsequent fluorescence. For example, plastics such as PET, PVC and PC are the most difficult to stain with NR and, accordingly, show weak fluorescent signals, being less hydrophobic materials (Enri-Cassola et al., 2017; Liu et al., 2021). Whereas *Lv et al., 2019* achieved strong fluorescence for these materials as well. Such discrepancies may be related to the very uncertainty of the concept of hydrophobicity of a particular type of plastic, such as the study by *Min et al., 2020* (***Fig. 2.5***) sometimes contradicts other researchers regarding the classification of some of the above polymers as less hydrophobic. However, the final hydrophobicity of the material also depends on the additives added to it (Kent, 2018). Accordingly, the color, as well as the shape of microplastics, also affects the final result in identification and counting. For example, such as black is unable to stain with NR, especially in the case of PVC (Shim et al., 2016). While fibers were considered the most difficult form to dye (Veitur et al., 2019; Tamminga, 2017). A study by *Veitur et al. (2019)* summarized the unsatisfactory result of dyeing polyester, which is currently the dominant contaminant. Which correlates with other results regarding the dyeing of PVC and/or polyester (Shim et al., 2016), which have similar properties but different names. Thus, a strategically important task is to find a dyeing method and a correct identification procedure for PVC and polyester, as well as other slightly hydrophobic plastics, which make up at least 25% of the European plastic demand in 2015 (Mickelaki et al., 2020).

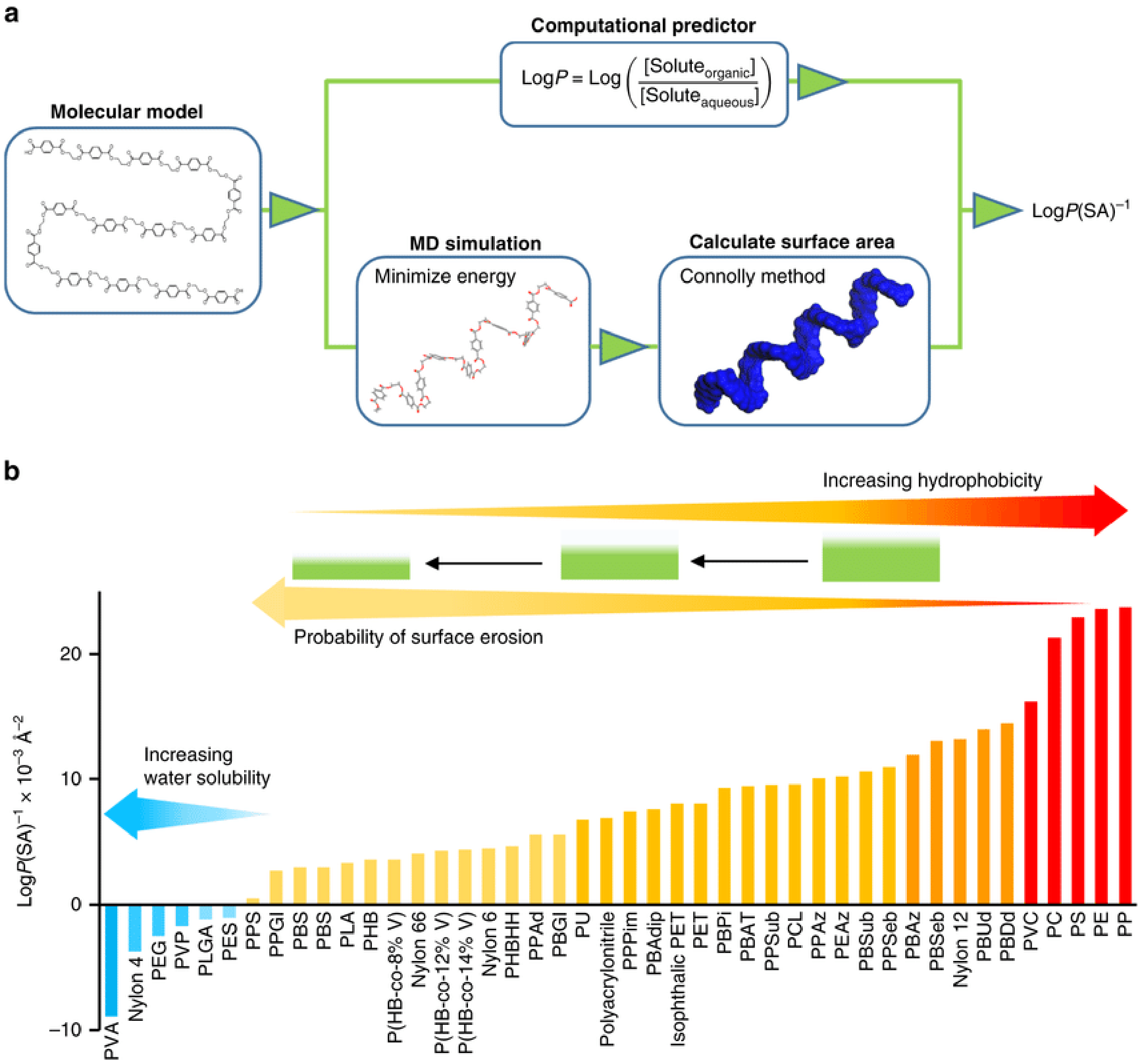


***Fig. 2.5***. *Plastics cover a wide range of hydrophobicity: a) Flow chart for calculating hydrophobicity; b) range of LogP(SA)$^{-1}$ values for various plastics. (source:Min et al., 2020)*

To all other, NR dye has a significant drawback - the ability to also stain organic objects, which is dictated by its nature and area of initial application. So the works of *Shim et al., 2016* and *Maes et al., 2017* state the possibility of a false-positive result when staining samples that come from natural objects with a high presence of nutrients, such as microalgae and mammalian cells, as well as tree species and biota (Sturm et al., 2021). Because of this, the overestimation when counting colored particles can reach about 1.4 times when the result is verified using FT-IR spectroscopy (Shim et al., 2016). In this regard, many researchers point to the need for the treatment step as a correct digestion, as a key factor in working with NR. ***Table 2.1*** provides an overview of the types of solvents previously used in experiments, which confirms the importance of this preparatory step for the identification process in general and in the application NR in particular. In support of this, Sturm el al., 2021 (among others) demonstrated a comparison between untreated and treated chitin particles when treated with two types of reagents: the commonly used $H_2O_2$ and $H_2O_2$ with the addition of chitinase to enhance the result of removing organic matter from the filter.

Scientists have tested other dyes and combinations such as: Rhodamine B (Tong et al., 2021); disperse dyes (iDye of different colors), Rit DyeMore Kentucky Sky (kentucky dye) (Karakolis et al., 2019); Safranine T, fluorescein isophospate (Lv et al., 2019), NR in combination with methylene blue MB (Michelaraki et al., 2020); NR in combination with DAPI (4',6-diamidino-2-phenylindole) (Stanton et al., 2019), etc.

# 3. CASE STUDY

## THE STUDY SITE

This study analyzes the central section of the Lido di Metaponto beach in the Basilicata region, which is one of the most popular beaches to visit during the summer season. For convenience, a small study area is named Metaponto 1 (***Fig.3.1***, ***3.2***) in the seaside resort of Metaponto, one of the most important on the Ionian coast of Lucania, part of the municipality of Bernalda (MT).

***Fig. 3.1.*** *Metaponto beach with the following coordinates 40°21'33'' N 16°50'13'' E*

***Fig. 3.2.*** *Closer satellite view (from Google Maps) of the beach under consideration*

This is an interesting place to study this problem. The reasons are: firstly, Italy is one of the most touristic countries in the world, and Basilicata is a southern region, which means it has a high level of beach visits, especially during the summer season; secondly, it is a peninsula washed by many seas. Both of these points are potential sources of pollution from both the sea and land.

In this case, the object under study cannot be 100% indicative for assessing the state of pollution, since it undergoes nourishment from time to time, due to its frequent attendance, as well as coastal erosion. And this means that the amount of plastic of various sizes (both micro and macro) is probably underestimated.

## SAMPLING

Sampling for laboratory analysis was carried out on 04/02/2021 and was carried out in accordance with the following accepted methodology and developed scheme (***Fig. 3.3***):

- 2 sampling points were identified within the town of Metaponto;
- for each point a 10 m long area has been identified divided into 3 zones:
    - Zone A - between the high and low tide line (close to water table outcrop);
    - Zone B - on the high tide line
    - Zone C - 15 from zone B
- at a depth between 2 and 5 cm, 3 samples were collected for each area, respectively at 2, 5 and 9 m from the point of known coordinates, for a total of 18 samples.

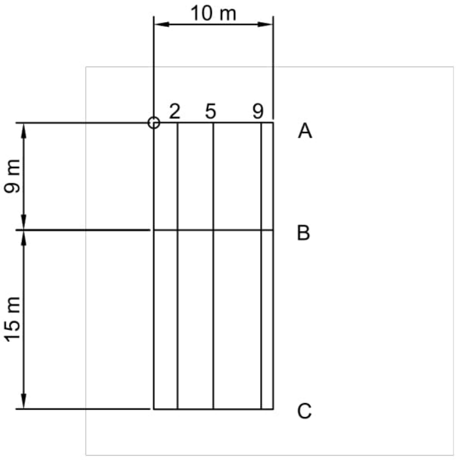


***Fig*. *3.3***. *Sampling scheme (zonation)*

The samples were collected (with the aid of a steel spoon) inside glass jars previously washed and cleaned with ethyl alcohol.
The identification codes of the samples contain a letter (A, B, C) which refers to the area and a number which represents the position with respect to the known coordinate point, ie METAPONTO1: 1,2,3 (***Fig. 3.4***). Then the obtained samples were stored in a refrigerator in the absence of light in order to exclude its destructive effect on plastic.

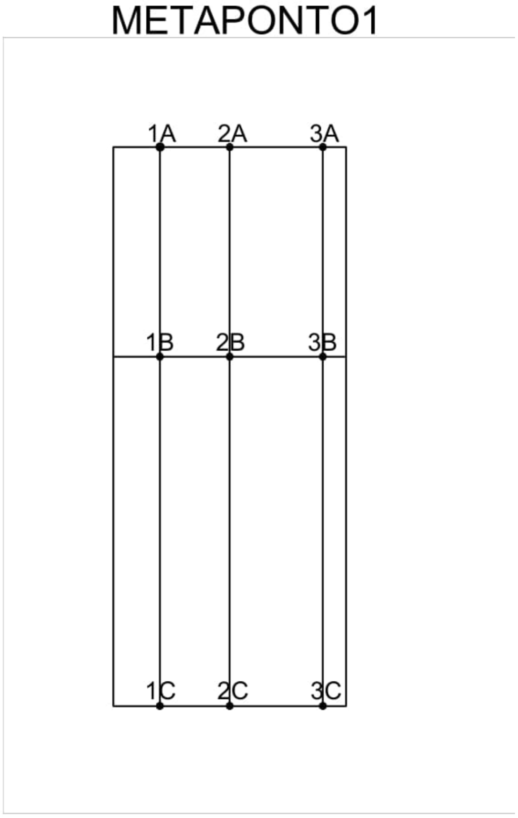


***Fig. 3.4***. *Sample numbering scheme*

The reason for choosing this sampling approach is the recommendation of *Hidalgo-Ruz et al. (2012)* according to which a comparative analysis of different sections of the beach should be carried out to determine the trend towards accumulation dynamics of microplastics. Some scientific papers, analyzed by *Hidalgo-Ruz et al.,* confirm that depending on the distance from the seashore (***Fig. 3.5***), different coastline zones of the emerging sector (supratidal and intertidal zone) accumulate and contain different types of plastic depending on from various environmental factors (wind, waves, etc.).

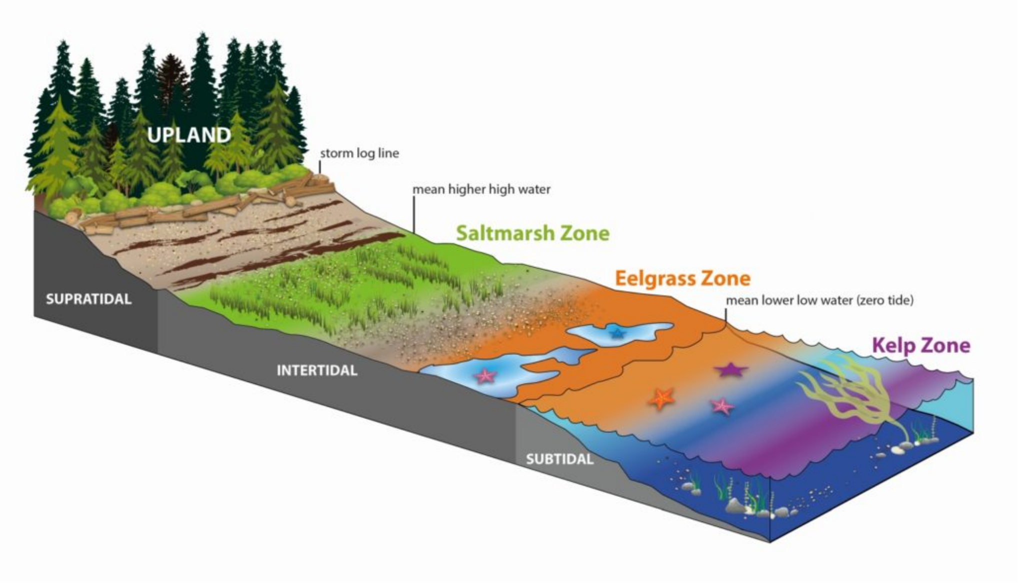


***Fig. 3.5****. Transversal profile of the beach divided into sectors (source: Project Watershed)*

The sampling method used in this paper is an adapted version of the standard method developed by *Bersley et al (2017)*, based on the analyzed and eligible 22 scientific papers on the identification of microplastics in coastal zones, according to which it is necessary to divide the beach area into four areas:
Zone A - between the high and low tide line
Zone B - on the high tide line
Zone C - 30 m from the dunes
Zone D - 15 m from the dunes
considering a sampling surface 100 m long. From each area 10 sand samples of approximately 50g (in dry weight) at 3 m, 14 m, 20 m, 32 m, 41 m, 45 m, 50 m, 59 m, 77 m, 98 m are taken, starting from a reference point at known coordinates, for a total of 40 samples (***Fig.3.6***). Since the sampling depth determines a different abundance of collected microplastics, it would be advisable to take samples at different depths (1, 2, 5, 10 cm) at the same sampling point.

The choice to use a different (i.e. adapted) method than that of *Besley et al* is due to several reasons:

- the practical difficulty in collecting a high number of samples (40) per sampling point and the consequent transport of them (from Basilicata to Turin);
- the presence of people involved in tourist / recreational activities made it impossible to temporarily isolate a 100m long stretch of beach;
- the Ionian coast, in particular the Metaponto area, has been subject to erosion for several years: bathing establishments are located a few meters from area C.

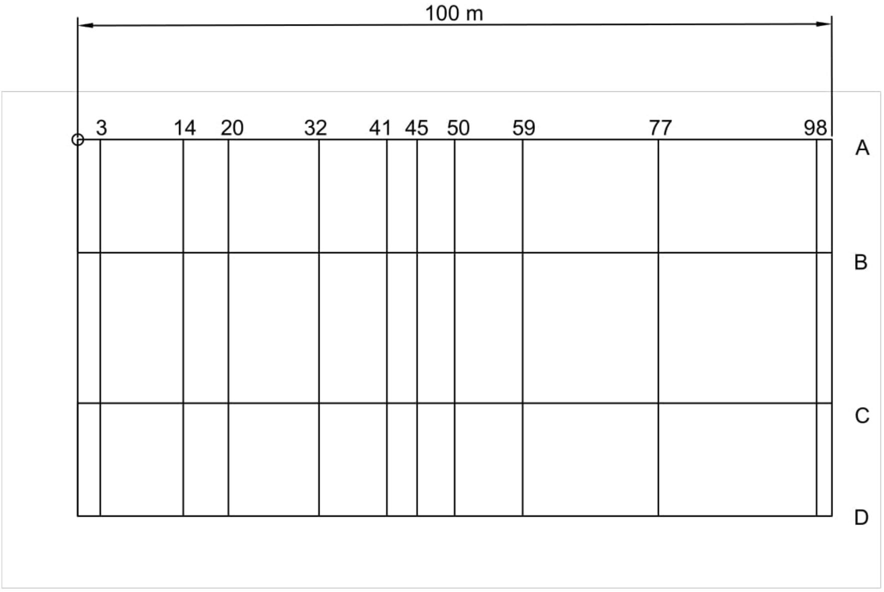


***Fig*. *3.6*.** *Scheme for sampling according to the method of Bersley et al (2017)*

## INVESTIGATION METHODS

The next step after taking material from the beach is the direct separation of microplastics from sand in the laboratory. However, some scientific papers describe the experience of in situ separation using a sieve of 5 mm or or smaller in the range of 10-300 micrometers as a pre-separation (Nguyen et al, 2019) to cut off larger and/or smaller fractions respectively, which are not the subject of study due to the discrepancy between the category of microplastics, i.e. less than 5 mm and more than 0.1 mm (if the subject of study is not the analysis of nanoplastics). In this case, this step was carried out in the laboratory.

In general, separation methods vary from work to work (as with all stages of microplastic identification and enumeration). However, the methodology recommended by *Hidalgo-Ruz et al (2012)* based on the analyzed numerous works, as well as the clearer guidance of *Balestra and Bellopede (2022)*, in combination with electrostatic separation for part of the material based on the results, was adopted as a guide to action from *Enders et al (2020)* research and also based on a successful procedure in similar experiments previously performed in this laboratory. The choice of an additional step in the form of electrostatic separation was taken as an experiment to determine its effectiveness for the subsequent possible use of this kind of methodology as a sand cleaner from microplastics directly on the beach on a large scale. Both approaches were applied in parallel on the same material from each line.

Despite the above strict sampling procedure, during the work it was necessary to combine material from three samples from each line, namely 1A, 2A and 3A were combined into sample A, the same was done with samples 1C, 2C and 3C due to insufficiency of material for subsequent procedures. Whereas samples 1B, 2B and 3B were subsequently not analyzed rather due to the close location of lines A and C, as well as the time frame.

Then both samples A and C were subjected to the following preparation procedure before subsequent separations:

- wet weighing in an aluminum mould;

- the material in an aluminum mold and covered with aluminum foil with small holes to prevent contamination was placed in an oven at 40 ° C for a day;

- re-weighing showing a significant reduction in weight due to evaporation, so the material spent more than a week in the oven, being weighed daily, until completely dry (when after 3 daily weighings no more variation in weight was observed);

- further, 400 grams were weighed and separated from the dried material, which were then divided into two portions: 200 grams intended for electrostatic separation (marked A (1) - for the line closer to the sea and C (1) - for the line located approximately in 24 meters from the sea (***Table 3.1***) and 200 grams for densimetric separation (similarly, following the same principle, labeled A(2) and C(2)). The instruments involved in the experiments (which are not plastic) were subjected to thorough cleaning with detergent, ethanol and an ultrasonic cleaner before each stage of the stage (ultrasonic cleaning tank was used to clean the screens before each sieving of the material to prevent cross-contamination of different samples).

## *Electrostatic separation*

Before the electrostatic separation procedure, a 1mm and 0.5 mm mesh sieving was performed. As the results of studies by Enders et al (2020) showed a high level of efficiency for beach sand contaminated with microplastics > 450 µm and ≥ 2 mm ( 99–100%), while for the range of 63–450 µm, the efficiency varied from 60 to 95%. Also, for material larger than 450 µm, there was no need to carry out a subsequent separation, since microplastics could be quite easily identified under a microscope, or even by the naked eye, which was one of the goals of this work. Unfortunately, in this case, it was not possible to confirm or refute this thesis, since almost the entire volume of beach sand, that is, 197.33 grams (out of 200 grams), had a granulometry of less than 500 µm. Thus, in the case of fine sand, separation technology using only an electric separator is not possible, since material smaller than 500 µm may contain microplastic particles invisible to the naked eye, and organic matter and the sand itself will interfere with identification even under a microscope. Therefore, it was decided to resort to subsequent densimetric separation, after electrostatic separation, according to the following approach (shown in ***Fig. 3.7***):

- after granulometric separation, the material was again combined (due to the impracticality of passing less than 3 grams through an electric separator) and weighed again (weight decreased by 0.05 grams);

- 199.95 grams of material was passed through the electrostatic separator (Prodecologia) at the following settings: 20 kV; 30Hz; 25% (drum speed) and 3 material fractions obtained: conductive, non-conductive and mix (compartment/container between conductive and non-conductive);

- the non-conductive part was collected, weighed and poured into a glass container (baker, previously washed and cleaned with ethanol), while the remaining 2 fractions were subjected to re-separation at changed settings (25 kV; 35 Hz; 25%) to increase the efficiency of material selection, containing plastic.

From two passes a total of 31.91 grams of non-conductive material was collected, which was then subjected to densimetric separation. While the conductive and mix materials were not subsequently included in the analysis (although it may be worthwhile to analyze these two samples as well when conducting similar work, for a more complete picture of the effectiveness of electrical separation for plastic in order to quantify how much plastic remained / could remain in two other subgroups).

Unfortunately, the first separation attempt spoiled material C(1), which was visible to the naked eye, due to insufficient pre-cleaning of the electrostatic separator despite all preventive cleaning measures (for sample A(1), the device underwent an additional rigorous cleaning).

## *Densimetric separation*

The preparation for the densimetric separation was also carried out by granulometric separation, but, this time, into more fractions, for the size classification of the plastic itself.
The grid cascade consisted of the following grid sizes: 1, 0.5, 0.250, 0.125 mm and the bottom, placed on an automatic vibro-separator for 10 minutes.

As demonstrated in the ***Table 3.1*** in the course of the experiment, for each category of both samples A and C, similar weights were obtained regardless of the distance to the sea, but quite different fraction weights (for example, A(2)_1+0.5mm and A(2)_0 .250mm). Since the size above 1mm was practically absent, it was decided to combine two classes, that is, 1 and 0.5mm.
According to the procedure developed by *Balestra and Bellopede (2022)* for method standardization, an equal amount of material should be taken from each class in order to be subsequently mixed with an equal amount of solution. In this case, it was impossible for some of the fractions, therefore, exactly 40 grams were separated from the classes with large weight categories (in accordance with the methodology in some studies in which these data were indicated, according to which 40 or 50 grams of material are generally dressed from total weight (Jahan et al., 2019; Enders et al, 2020), while the low weight classes were left as is. Then, a saturated solution of sodium chloride (NaCl) was prepared, dissolved in distilled water, at the rate of 358 grams per liter of water or 200 grams of NaCl / 0.6 L $H2O_{distilated}$, using an magnetic stirrer until the NaCl was completely dissolved and a density of 1.22 was obtained.

***Tabel 3.1.*** *Marking of subsamples and their quantity after particle size separation*

| Sampling zone | after Electrostatic Separation | after Densimetric Separation | | | |
|---|---|---|---|---|---|
| **Closer to the sea** | *A (1)*<br>31.91 g | *A (2)*_1+ 0.5mm<br>2.69g | *A (2)*_0.250mm<br>101.24g | *A (2)*_0.125mm<br>95.8g | *A (2)_bottom*<br>0.24g |
| **Further from the sea (~ 24 m)** | *C (1)* *<br>46.91 g | *C (2)*_1+ 0.5mm<br>2.9g | *C (2)*_0.250mm<br>111.6g | *C (2)*_0.125mm<br>86.12g | *C (2)_bottom*<br>0.22g |

** lost due to contamination in the ES*

Then each sample obtained was subjected to the following procedure:

-adding the solution to the dry sand fraction, at a ratio of 100 ml per 10 grams in the case of samples with sufficient weight (i.e. only 400 ml per 40 g) and about 40 ml in the case of low weight in order to sufficiently cover the material and ensure adequate thickness of the water layer;

active mixing of the resulting substance with magnetic stirrer for 8 minutes for samples containing 40 g of sand and 2 minutes for samples with a lower weight;

- the resulting mixture was left to stand for at least 8 hours (i.e. one night);

- the upper layer of the settled liquid, potentially containing floating microplastics (supernatant), was taken with a graduated pipette and aspirator bulb (12 times 10ml for 40 g samples and once 10ml for less than 40g), and then sieved through glass microfiber filter (Whatman, 1.2-μm pore, 47 mm) placed on a vacuum filter (so-called Büchner filter);

- the resulting filter with the filtered material was placed on a cleaned glass Petri dish, covered with perforated aluminum foil and placed in a forty-degree oven for at least 4 hours.

Some used devices for this stage are shown in ***Fig. 3.8.***

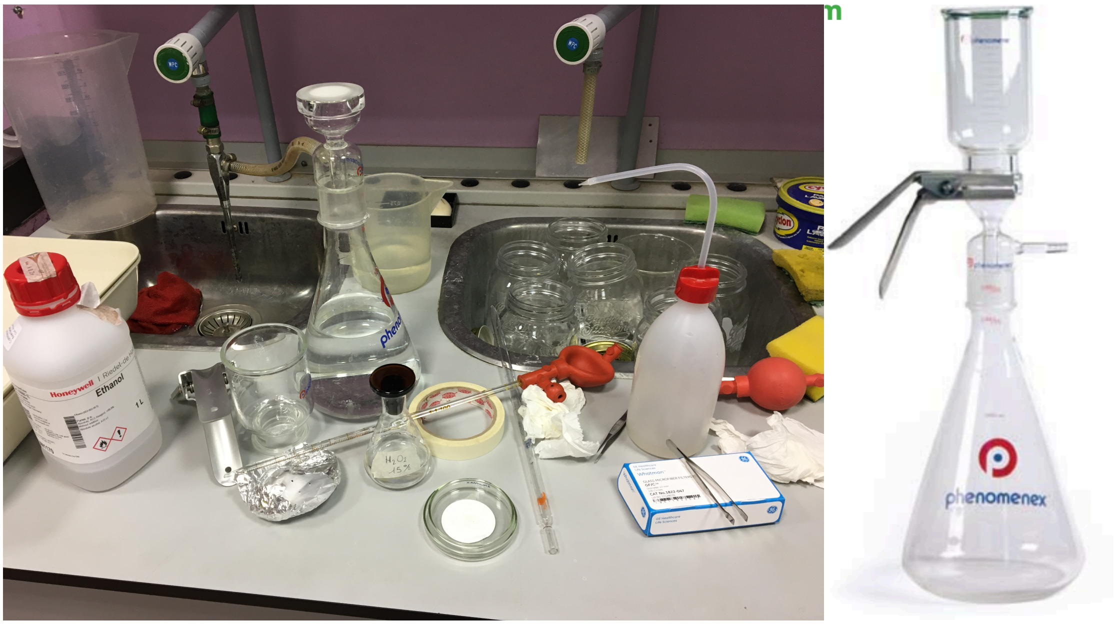


***Fig. 3.8**. Some tools used in densimetric separation*

## *Organic matter removal*

The stage necessary to eliminate organic matter from the filter was carried out according to the following scheme:

- 0.5 ml of hydrogen peroxide ($H_2O_2$) with a concentration of 15% was added to each of the obtained filters using a pipette;

- then left for at least 30 minutes to react with the material contained on the filter;

- and finally placed in an oven for 1 hour until completely dry at 40 ° C.

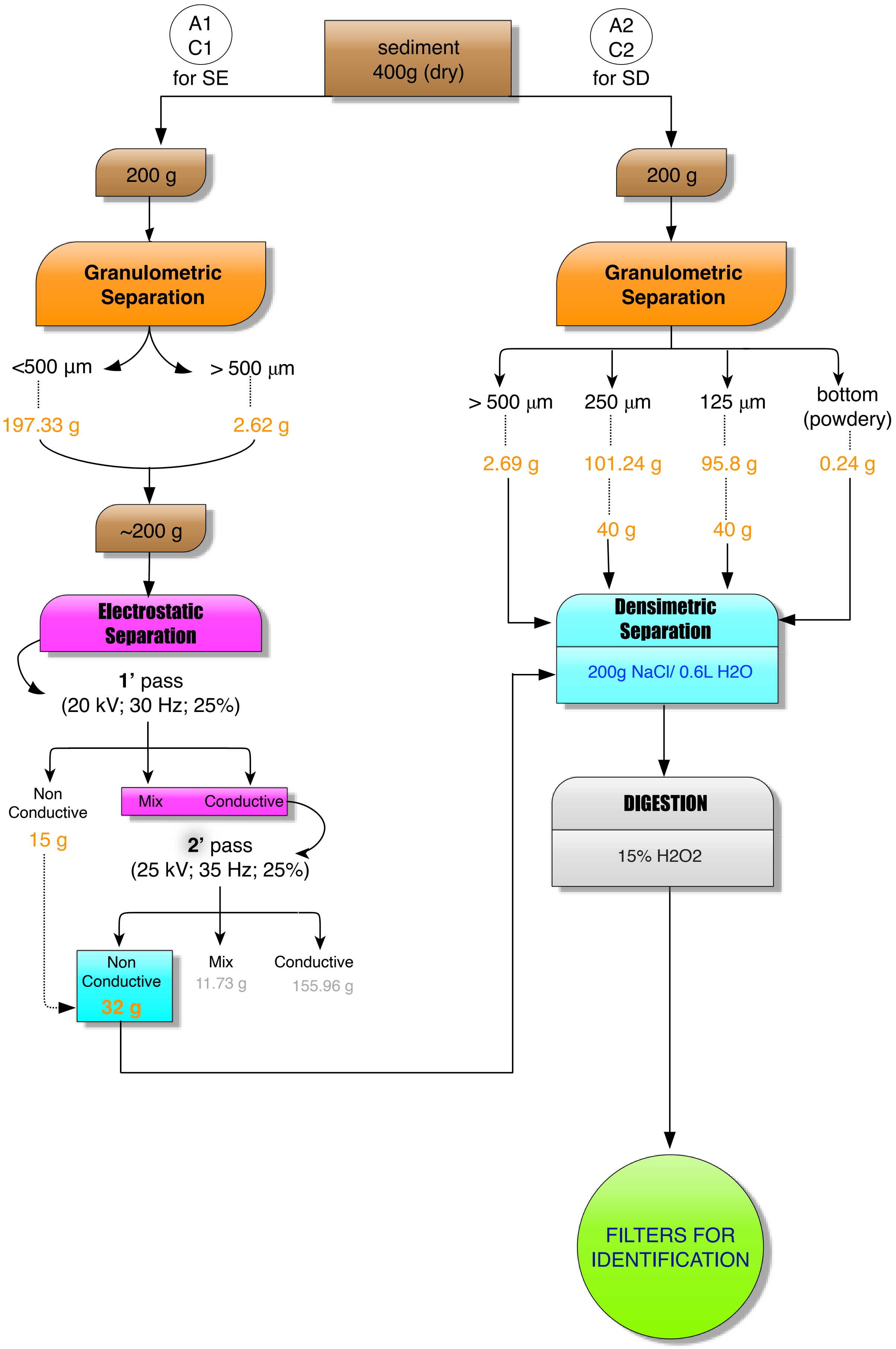


*Fig. 3.7. Flowchart of separation process in this study*

## *Microplastics identification*

### ➢ **Optical microscopy with UV**

In accordance with the procedure and recommendations of *Balestra and Bellopede, 2022* for the visual identification using a UV microscope, the following devices were selected and used:

- Optical microscope Leitz ORTHOLUX II POL-MK with 2.5x, 4x and 10x objective lens;
- Digital camera DeltaPix Invenio 12EIII with12 Mpx sensor assembled to the microscope;
- UV lamp Alonefire SV10 365 nm (UV flashlight 5W);
- Mini Portable LED Flashlight.

#### ***a) preliminary test***

Due to the ambiguity of both classical criteria themselves, subsequently questioned by later scientific articles and / or visually not corresponding to them (Norén et al, 2007; Curren et al, 2020; Limbago et al, 2021; Windsor et al , 2019; Mukhanov, Vladimir & Daria et al 2019; Cole et al, 2014; J. Li et al 2015; Cheang et al, 2018; Stand et al, 2015; Pequeno et al, 2021; Curren et al, 2020; Ren, Peng Ju & Dou et al, 2020; Irfan, Tahira & Khalid et al, 2020; Turner et al, 2019; Picó & Barceló, 2019; Cincinelli et al, 2017; Pradit et al, 2020; Blumenröder et al., 2017), and due to conflicting conclusions and/or results in other scientific papers, an additional preliminary experiment was conducted to determine the visual characteristics of the fibers before and after their destruction. The goal of this experiment was to understand how the fibers of synthetic and organic fabrics can change when exposed to external forces, such as abrasive forces, which can't be exposed in the environment when interacting with various natural forces. In many works visual examples (photographs of detected MP samples) sometimes partially, or not at all, corresponded to the classical criteria; at the same time, the results of such works do not cause as much doubt as the criteria themselves, since they were confirmed not only by visual technology, but also by more advanced ones using various analytical identification tools such as FT-IR, Raman Spectroscopy, SEM (Scanning electron microscope) capable to recognize the type of plastic or other objects as well.

This mini-experiment was not intended to recreate the complex and variable environmental conditions and processes that plastics can undergo. In this case it was only a rough approximation of potential damage, especially in case of sand samples, since sand itself is one of the strongest and most effective abrasive materials used everywhere in various industries (for example, in industries for surface treatment metal sandblasting); moreover, it may be able to cause significant damage to plastic or, possibly, change it beyond recognition in natural conditions.

In this experiment, samples of several threads with the following composition were used:

- Sample 1: 70% polyester (trasparent), 30% cotton (blue)
- Sample 2: 100% polyester (green)
- Sample 3: 64% poliester, 36% poliuretan (white)
- Sample 4: 100% polyester (yellow)
- Sample 5: 100% polyester (black)

Each of the above samples were analyzed under a microscope before and after destructive impacts. The destruction of the fibers occurred using the impact of the edge of the metal tweezers on the samples and tearing movements from side to side. The results of such influences are shown in ***Figures 3.9, 3.10, 3.11, 3.12, 3.13***.

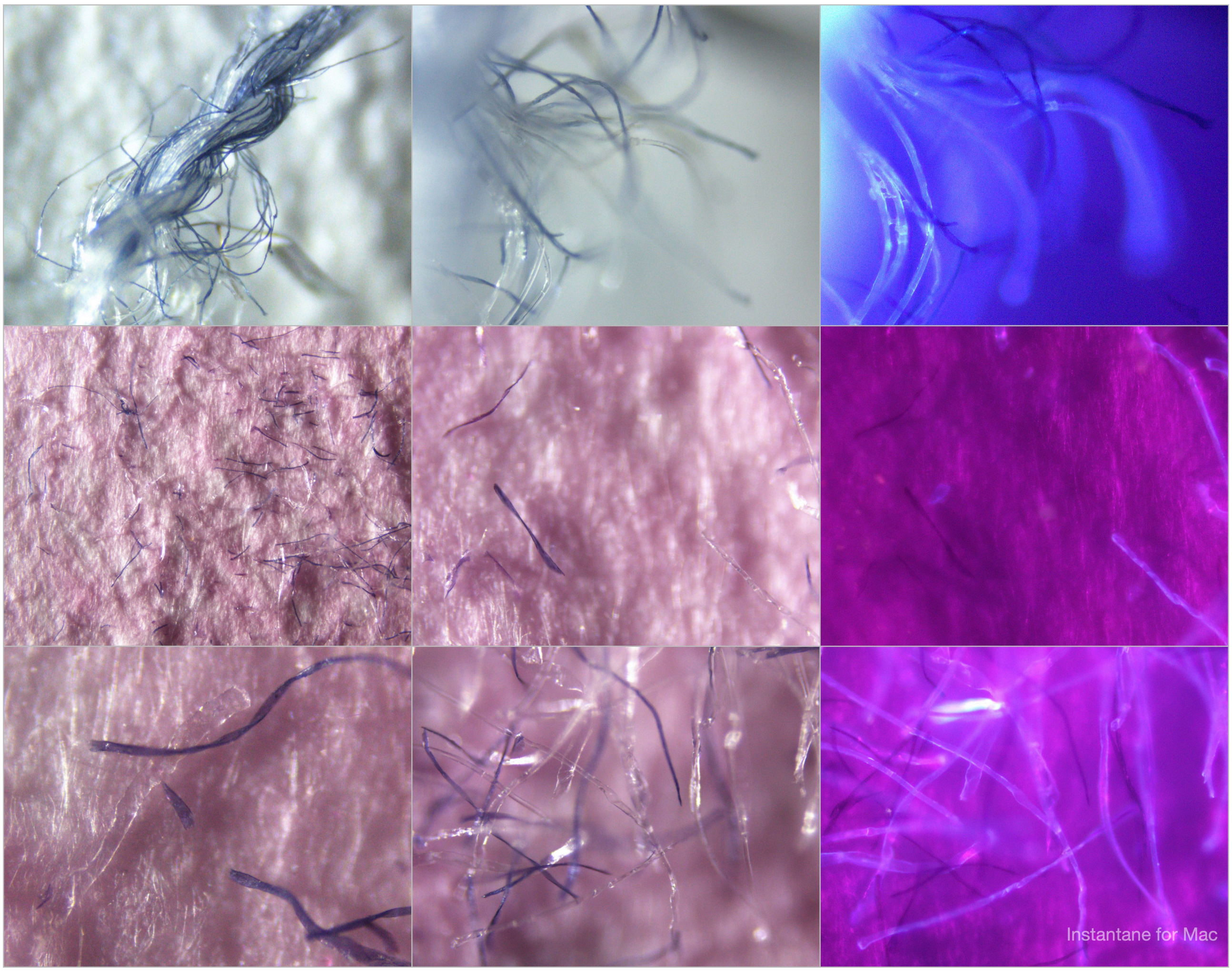


***Fig. 3.9.*** *Sample 1: before (above) and after (under) destruction of fiber (transparent polyester and blue cotton) under LED (left) and UV light (right)*

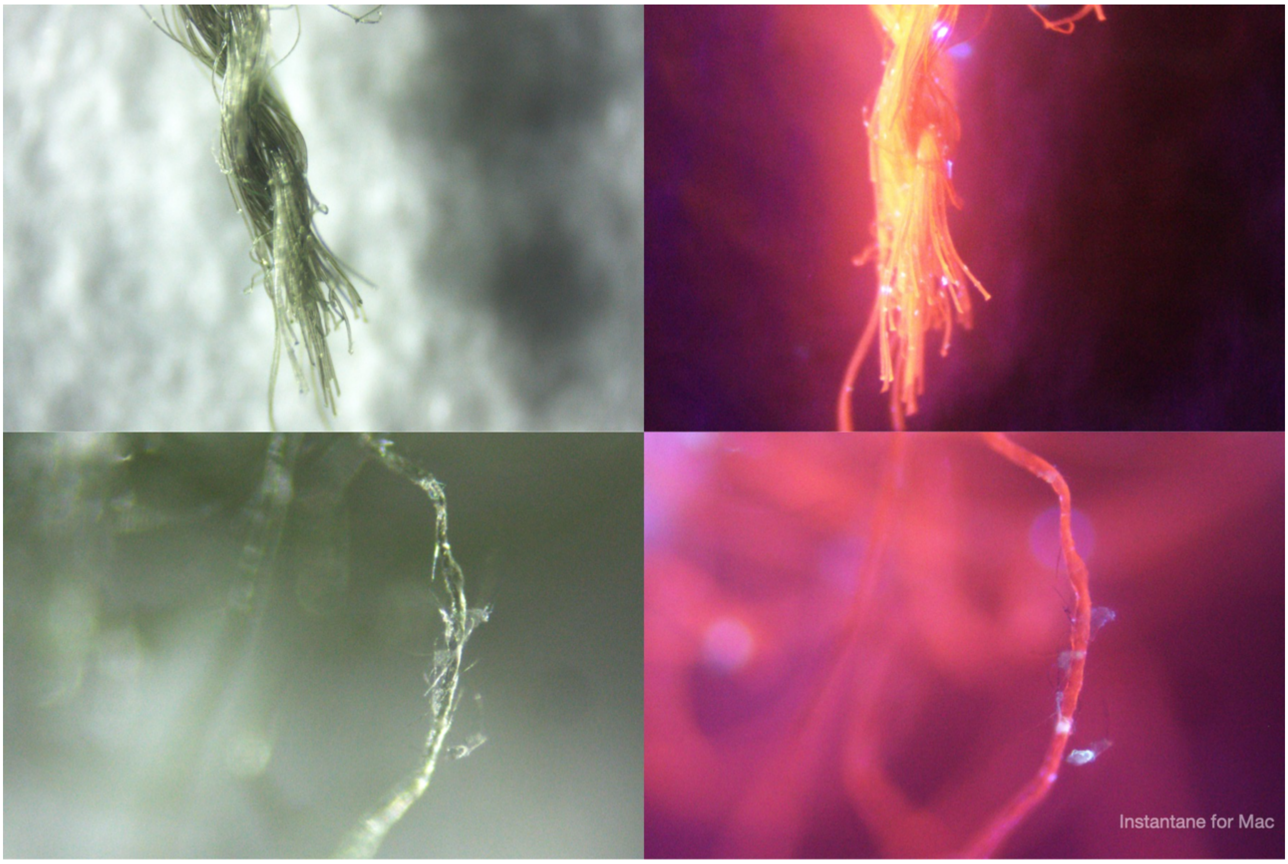


***Fig. 3.10.*** *Sample 2: before (above) and after (under) destruction of fiber (green polyester) under LED (left) and UV light (right)*

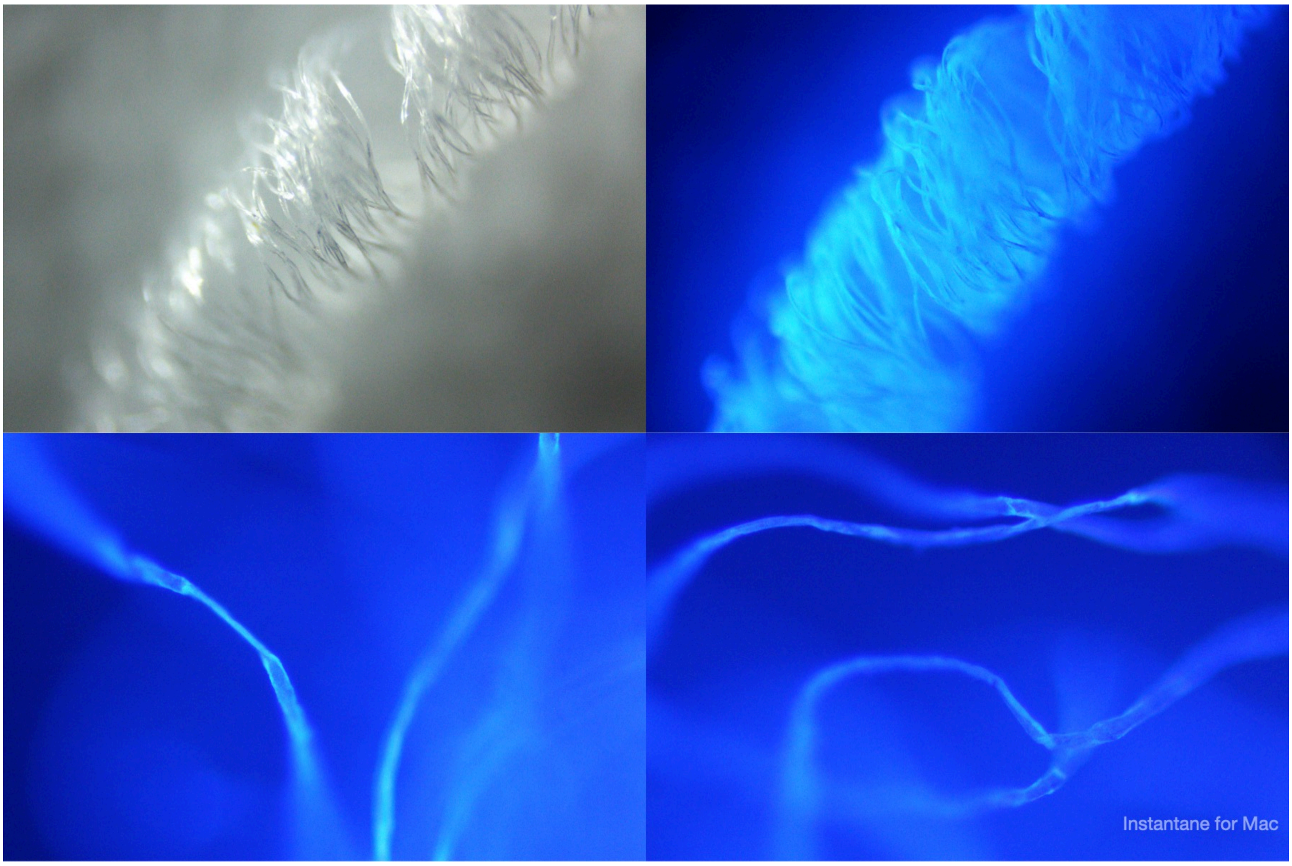


***Fig. 3.11.*** *Sample 3: before (above) and after (under) destruction of fiber (white poliester+poliuretan) under LED and UV light*

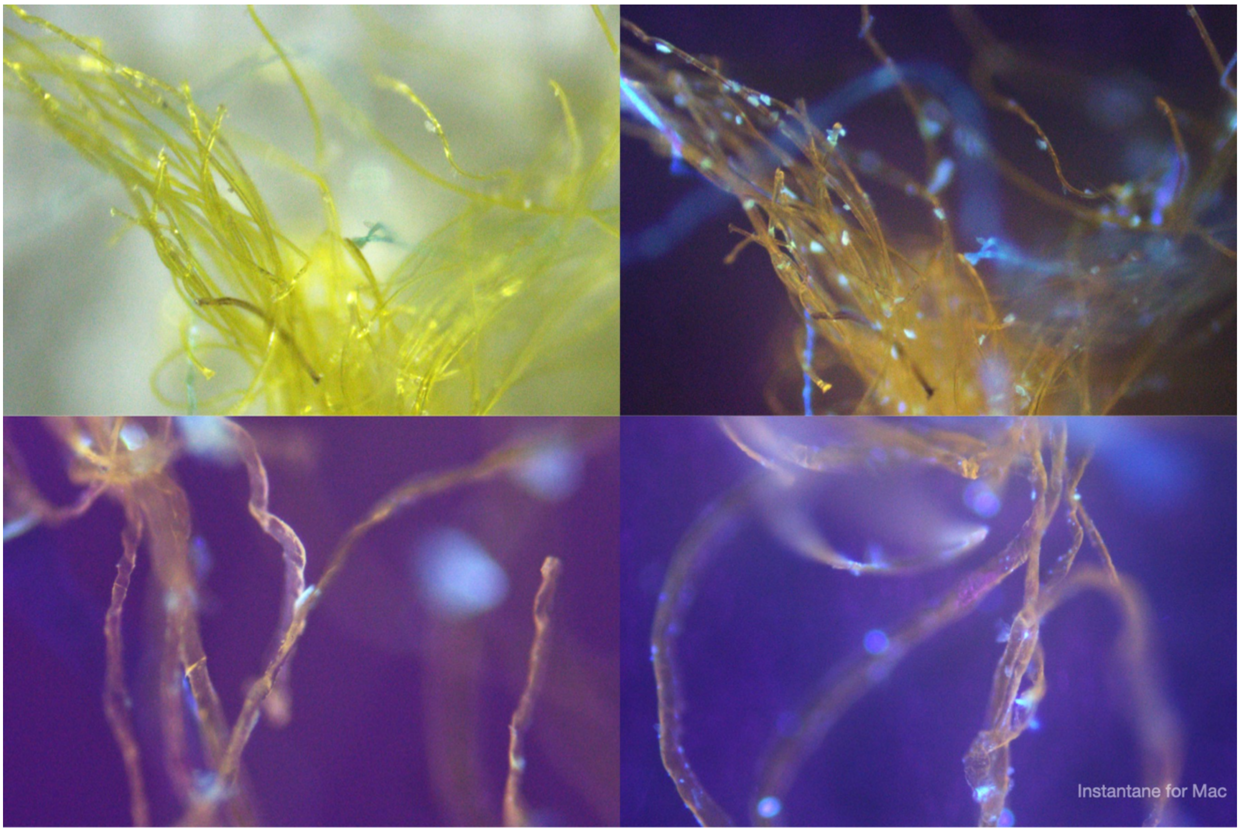


***Fig. 3.12.*** *Sample 4: before (above) and after (under) destruction of fiber (yellow polyester) under LED (left) and UV light (right)*

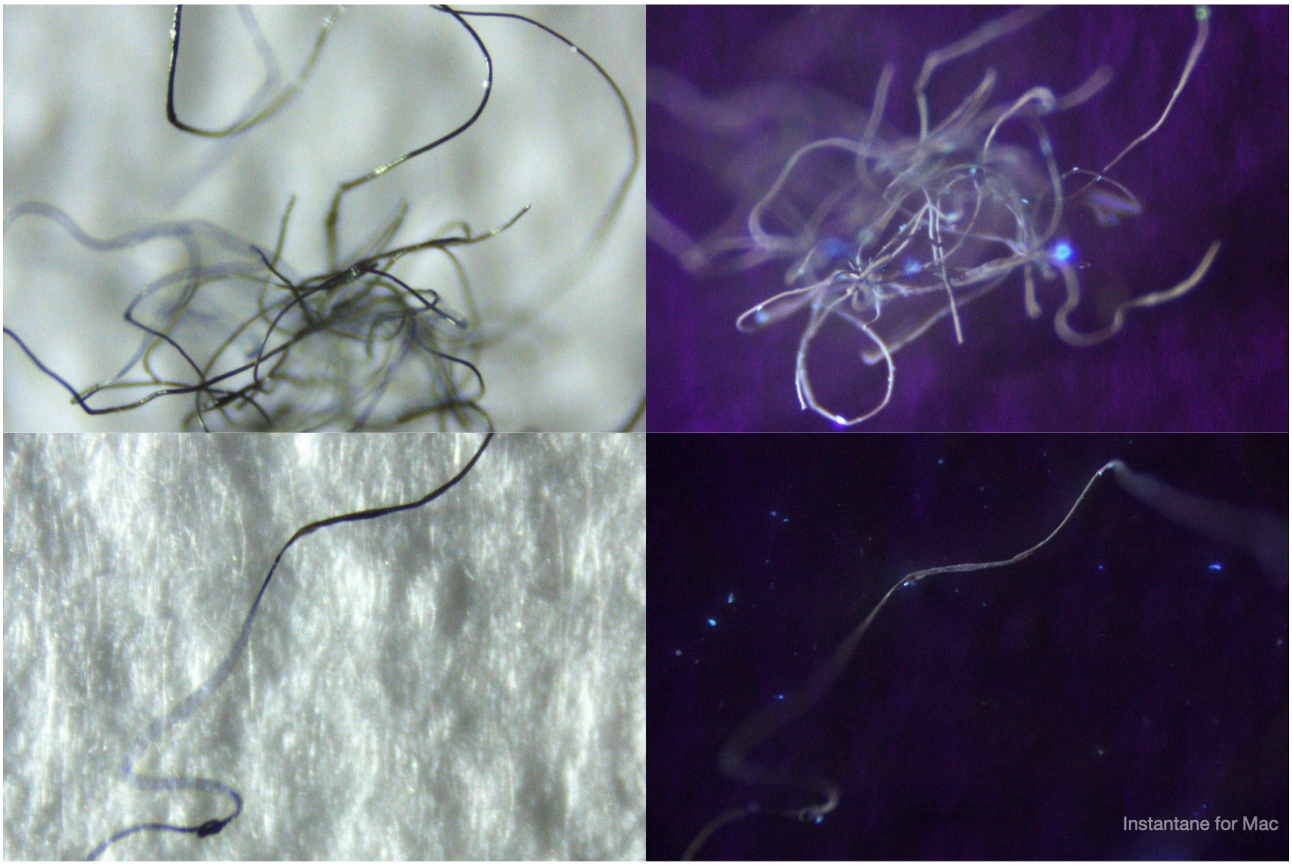


***Fig. 3.13.*** *Sample 5: before (above) and after (under) destruction of fiber (black polyester) under LED (left) and UV light (right)*

Some samples had a clear and uniformly distributed color, which correlates with the result obtained in this experiment: the cotton fiber of Sample No. 1, despite the damage caused, practically did not change and did not lose its blue color, while synthetic fibers of all samples underwent significant changes in shape. While the color loss is not noticeable on the tested synthetic materials with this type of damage, however, sample No. 2 (***Fig. 3.10***) demonstrates a partial loss of fluorescence at the edges following the detachment of the material.

In turn, all synthetic samples demonstrate greater or lesser deformation, the ability to flatten and tie into knots, to bend by 90 degrees, delamination, twisting and to change the original shape significantly, becoming more and more similar to natural fibers and to the parameters that are attributed to them.

### b) analysis of filters

Despite the above-described ambiguity of the criteria (*Microplastic identification and quantification methods: critical review*) and results of the preliminary test, it was decided to analyze the previously obtained samples on filters with visual identification under UV microscope, basing the identification and final count of the MPs found particles mostly on conservative criteria of *Norén et al, 2007*. The threshold identification limit was set at 0.1mm (Balestra and Bellopede, 2022; European Commission (2013)), below which particles found were not counted due to the resolution of the microscope. Identification used primarily a 2.5x lens, but typically 4x and 10x lenses were also used to improve visual evaluation. Each filter was divided into a matrix consisting of 5x5 mm segments, in which microplastics were identified and counted (***Fig. 3.14***).

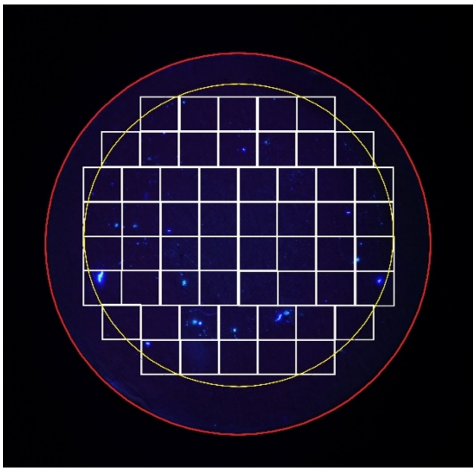


***Fig. 3.14.*** *Counting areas on filter (rectangles). Red circle defines the filter area, yellow circle defines the filtered surface. (source: Balestra and Bellopede, 2022)*

The more general concept of "microplastic" has been broken down into more specific categories according to the shape and size (greater or smaller than 1 mm) of the particles, as recommended by *Balestra and Bellopede, (2022)*, *Crawford and Quinn (2016)*, namely: fiber (bigger than 1 mm) and microfiber (smaller than 1 mm), film and microfilm, pellet and microbeads, fragments and microfragments, foam and microfoam.

In general, there is no single standard categorization of forms (Wu et al, 2018) and among scientific works, both different set of categories are used, and their own are invented or more general ones are specified for specific research goals, such as one category "fragments" *Song et al, (2015)* highlighted additional ones, such as paint particles and plastic, depending on its type as it helps in analytics of its source. Also, *Cowger et al (2019)* proposed an additional category, in order to separate "fibers" from "line" since they have different origins and are often confused or written into the same category, despite the fact that the fibers are related exclusively to the garment, whereas line are oblong fragments that may be very similar to fibers, but they are not. Another necessary category was proposed by *Campanale et al 2019*, namely "flackes" for materials such as particles from rubber tires, which often have a specific shape, namely flakes of different shapes and thicknesses and, therefore, hardly belong to other categories; he similarly divided lines and fibers, accompanying this categorization with an explanation of the possible origin of straight particles from fishing nets and fishing lines, and fibers from synthetic fabrics. Therefore, additional categories were included in this study.

### ➢ Nile red

After analyzing most of the currently available works that tested the Nile Rad dye as a microplastic identifier, a number of preliminary experiments with the use of this dye were carried out. The work of *Maes et al (2017)* and *Hengstmann et al., (2019),* was chosen as a reference point for light radiation, since only a microscope with a UV lamp was available in the laboratory, and not a green filter, in combination with the fluorescent microscope, could be numerically established as the most effective in carrying out of this kind of detection, and also because of following the initial strategy of detecting microplastics by use of methods and tools that have already been used previously in order to test their suitability for a new type of identification.

The most optimal concentration was 1 mg of dye per 1 ml of acetone as the most affordable, environmentally friendly, non-toxic and simple solvent (***Fig.*** *3.15*). This concentration was selected in accordance with some works that demonstrated the increasing effectiveness of the dye with increasing concentration (Sturm et al., 2021) and/or successfully tested this concentration (Fischer et al., 2016; Veitur et al., 2019; Hengstmann et al., 2019; Tamminga et al. 2017; Shim et al., 2016). With the help of a pipette, several drops of this composition were distributed on each of the glass filters so as to color only the various material previously placed on it for research, and then left for about 5 minutes for the reaction, and then placed for 30 minutes in a drying oven at 40 ° C. Then they were analyzed under a microscope with a UV lamp and under normal LED light. The step of washing after the furnace was deliberately skipped because it contradicted one of the goals of this study – verification of the ability of the contrast base to improve the identification of microplastics.

***Fig. 3.15.*** *Nile Red/acetone solution of 1mg/ml concentration*

The results of experiments on various characteristic types of plastic, natural and synthetic fibers, as well as the sand itself from the beach where the selection was carried out, are demonstrated in ***Fig.*** *3.16, 3.17, 3.18, 3.19*. Photos of the results were taken using the previously determined microscope and camera using LED light and UV flashlight (365 nm). These preliminary experiments were conducted in order to test how the NR dye behaves on different types of plastic and other surfaces whose composition is known in advance and thus facilitate subsequent analysis of filters on which microplastics and other not always identifiable objects were detected.

As the initial plastic materials, the following were selected: plastic glass, food film, expanded polyurethane foam sheet and various parts of the water bottle (lid, bottle itself and label). The study of the staining of the bottle components was of particular interest, firstly, because of the high prevalence of this type of packaging (and, accordingly, subsequently becoming one of the prevailing types of waste), and also because the bottle body consists of the PET (polyethylene terephthalate) material, which is also used for the production of synthetic fabrics, but having a different name, that is, polyester, which accounts for more than 60% of the world PET production; whereas packaging production – 30% (New Life Plastics Ltd). In ***Fig.*** *3.16* plastics of the following types: polystyrene PS (cup), polyester (fiber), HDPE (lid from the water bottle), PVC, PE (food film), polyethylene foam PEE (sheet), PET (water bottle), have different degrees of color or do not have it at all, as well as different degrees of fluorescence when using UV lamps. That is, polyester and PET were only partially stained and changed/increased their fluorescence at cut/damaged areas (on the edges), while HDPE did not stain or change its fluorescence at all. In turn, materials such as PS, PEE and PE have become heavily stained, changing color from transparent and white to a bright pink, easily recognizable in normal light; as well as under UV illumination, the fluorescence color changed from blue to yellow, orange and red, and the fluorescence intensity also increased significantly.

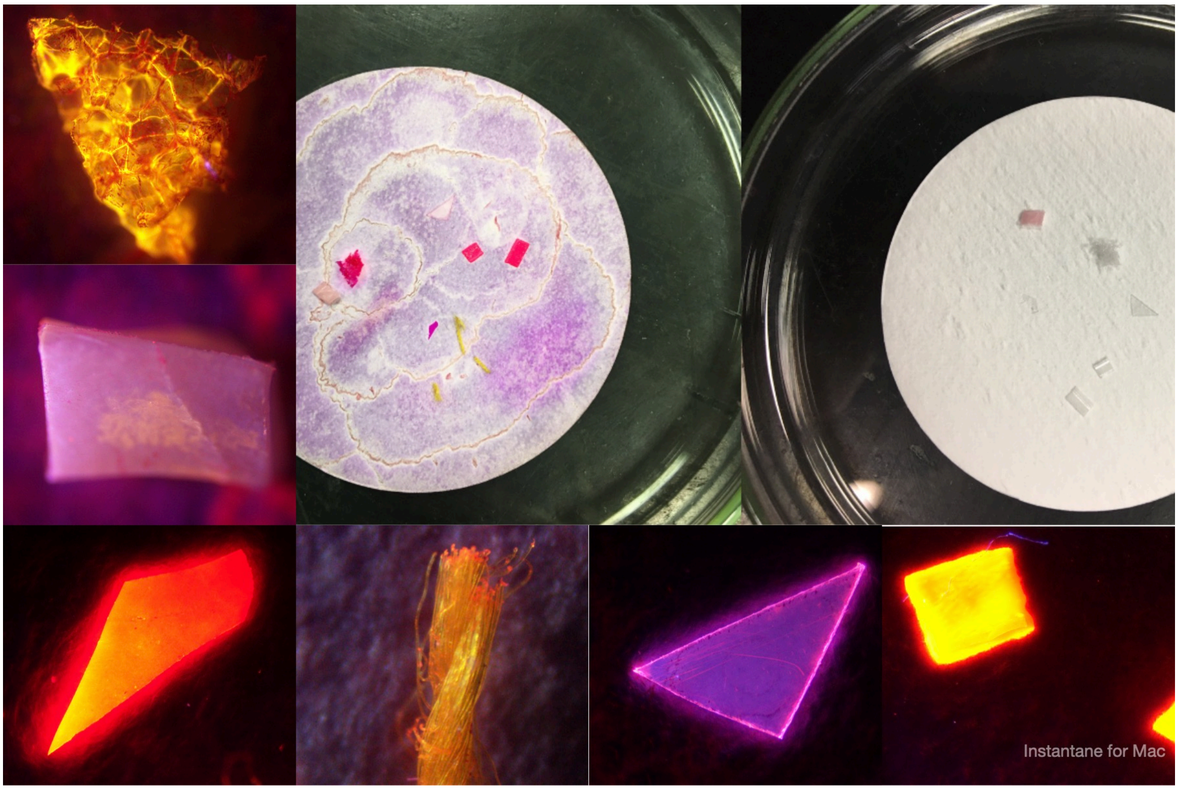


***Fig. 3.16****. Plastic particles before and after Nile Red staining with and without UV light under LED and UV light: a) foam PEE; b)HDPE; c) PE (food film); d) poliester (fiber);e)PET; f)PS*

Similarly, natural and synthetic fabrics of different compositions were also dyed, namely: white viscose, cotton and some synthetic threads used in the previous experiment (with destruction) such as transparent polyester + blue cotton and white polyester. As demonstrated in ***Fig.*** *3.17* and ***Fig***. *3.18.* both synthetic and natural white fibers have been dyed pink in daylight (except for the blue cotton, which has not changed color). However, when exposed to a UV light, the following features were noticed: synthetic fibers did not change either color or fluorescence intensity, except in places where the material was damaged (at the edges); viscose did not change its fluorescence even at the cut points; while cotton has acquired a pink glow under the UV light as well.
Also, it was decided to repeat the previous experiment with the destruction of these materials and then color them, due to the greatest presence of fibers in the samples (based on data from scientific articles). As a result, it is noticeable that:

- when destroyed, synthetic fibers are visibly stained and fluoresce very intensely, while cotton fibers are worse and, moreover, with strong destruction, cotton fibers reduce their ability to be dyed; however, this does not apply to dark blue cotton fibers that have not lost color, have not changed color and fluorescence, and are also slightly damaged. In turn, the viscose did not change the fluorescence before or after the destruction, but only slightly changed the color, which was not noticeable after the destruction of the fibers;

- destructive effects can be so strong that the fibers completely change their appearance and become unidentifiable as fibers, and more like a film.

Therefore, as can be noticed, the more an object receives damage of a different nature, the more it looks like other objects that are not microplastic, and less it meets the classical criteria that describe plastic. In addition, it was noted that synthetic fibers were much more easily destroyed than cotton fibers when the same force was applied and the same objects were used. However, some natural fibers (depending on their type and color) are capable of being dyed and in order to correctly interpret the identification of the material, it is also necessary to resort to certain criteria, such as the absence of strong and frequent twisting in a spiral around its axis, a strong plane /thinness of the fiber along the entire length, and the presence of a cellular or linear structure.

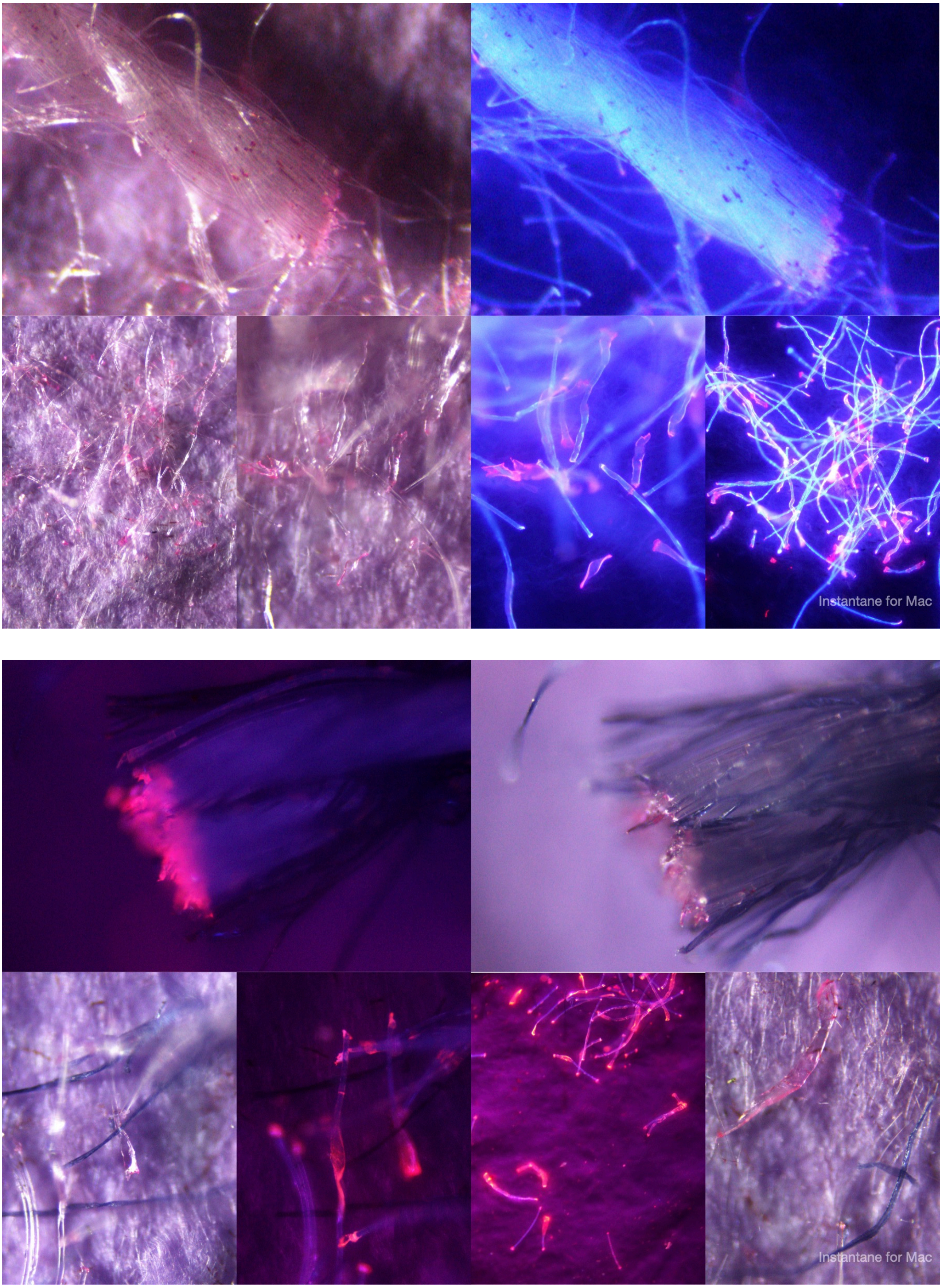


***Fig. 3.17.*** *Plastic fibers before and after destruction procedure and before and after Nile Red staining under LED and UV light: white polyester (above) and transparent politer, blue cotton (under)*

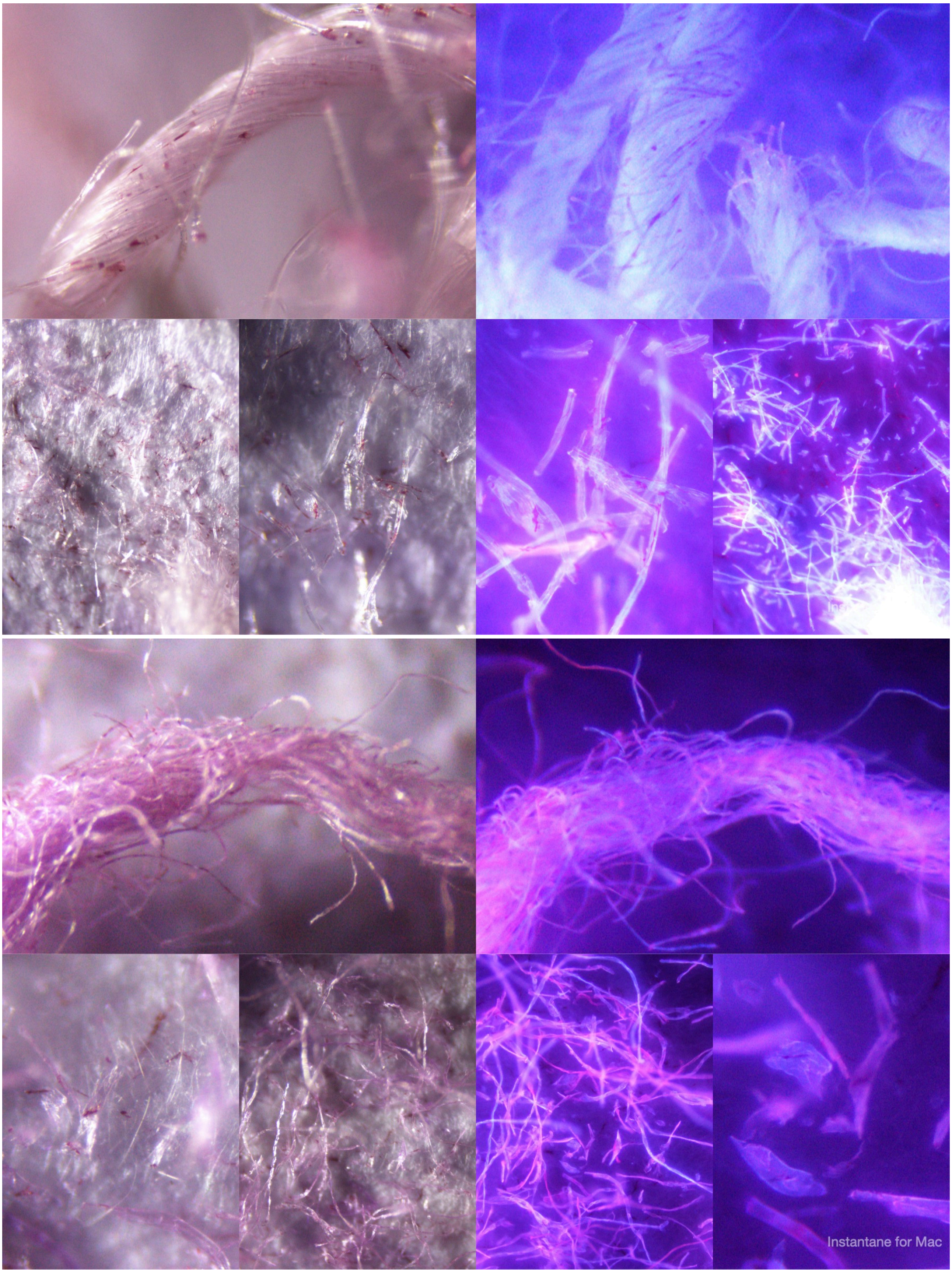


***Fig. 3.18.*** *Natural fibers before and after destruction procedure and before and after Nile Red staining under LED (left) and UV light (right): white viscose (above) and white cotton (under)*

Then the sand itself was subjected to the last experiment to see how the dye behaves on objects of mineral origin, due to their large presence on the filter and their similarity to microplastic fragments, which made identification very difficult and could lead to erroneous results (most likely to underestimation of the amount of microplastic). As seen in ***Fig**. 3.19*. minerals are not subject to staining, with the exception of some specimens on which the surface adhesion of the dye is visible. But despite this, the intensity of fluorescence of minerals, both colored and not, did not increase under UV illumination

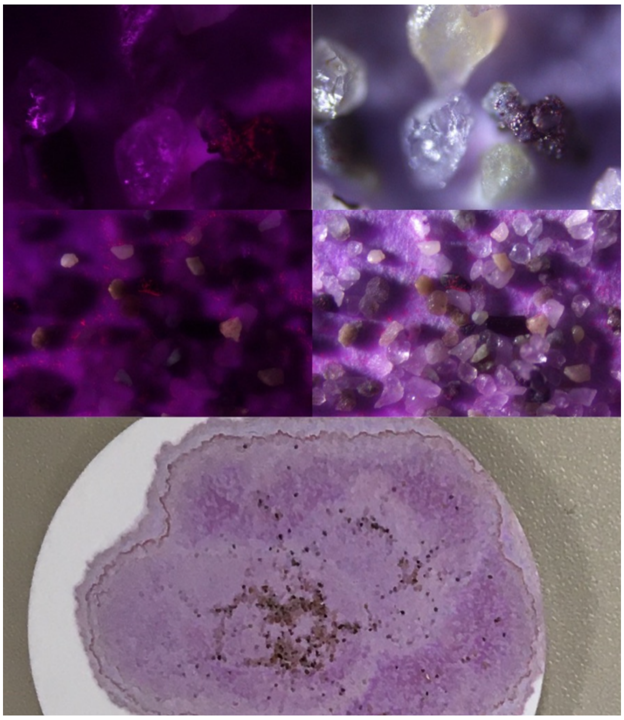


***Fig. 3.19**. Mineral material (sand) before and after Nile Red staining under UV (left) and LED light (right)*

As part of the staining experiment, another dye was also tested, namely Safranine with the chemical formula $C_{20}H_{19}N_4Cl$, based on the results of *L. Lv et al., 2019*. This dye is widely used in various industries, including biology for microscopy purposes. For this test, plastic materials from a previous experiment were taken, as well as several new types of polymers (whose chemical composition was not known).

Additional protective measures were taken to prepare the dye solution because Safranine is a toxic material. Also, unlike Nile Red, this dye was mixed with acetone in a smaller proportion, which is, 0.1 mg / ml (***Fig.** 3.20*), as it has stronger coloring properties (the rest of the procedure was carried out similarly to staining with Nile Red). However, experiments have shown (***Fig**. 3.21*) that Safranine stains most synthetic surfaces less successfully and does not change/increase their fluorescence intensity compared to Nile Red. And moreover, it makes the coloring result unobvious, because it paints the filter surface in the same color as the objects on it without any contrast between them. This ambiguity is especially pronounced with transparent surfaces, as in case of PVC. For example, when it is difficult to understand whether the transparent object is colored pink, or the color of the filter itself, or both. In order to make sure of this, it is necessary to move the object aside with tweezers or completely move it to the unpainted part of the filter, which is practically impossible in case of microplastics less than 500 micrometers in size, or to preliminarily carry out an additional washing of the filter with distilled water.

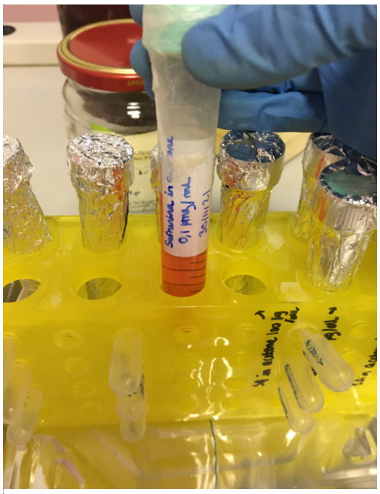


***Fig. 3.20**. Safranine/acetone solution of 0.1μg/mL concentration*

Overall, this dye has not been proven effective for use in these studies. However, it is possible that when using a different solvent, such as chloroform (toxic), ethanol, etc., the stain solution with Safranina will behave more effectively. Also, when using this dye, it makes sense to follow protocols that include a filter washing step to get rid of the dye absorbed by the filter (Liu et al., 2021).

***Fig. 3.21.*** *Plastic particles before and after Safranine staining with and without UV light under LED and UV light*

After carrying out the above experiments, the last to be stained were the filters with an objects previously found on them, identified (using a UV microscope) as microplastics.They were found in sand samples from the beach. Of all the prepared and previously analyzed filters, only 2 were subjected to staining and comparative before/after analysis. Namely, filters marked *A(2)_1+0.5mm* and *A(2)_bottom* were selected as samples with the most diverse and interesting objects found on them identified as microplastics, as well as objects that could potentially still be microplastics (but were not counted in visual identification using a microscope). The above filters were coated with a solution containing Nile Red dye, similar to that used in the experimental part and according to the same procedure, with the only difference being that in this case the entire surface of the filter was colored (applied 6-7 drops each). The result of staining half of the filter after it has dried in the oven is shown in ***Fig.3.22***

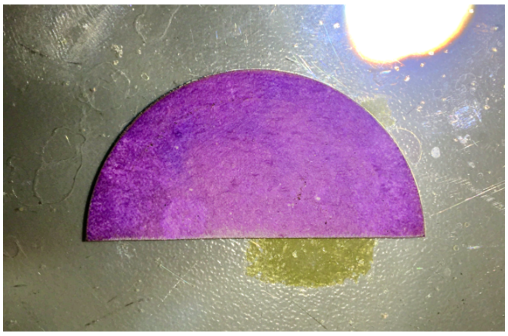


***Fig. 3.22.*** *One of the filters with filtered subsamples*

# 4. RESULTS AND DISCUSSION

## SEPARATION

As a result of granulometric separation, 98.69% (197.33 of 200 g) of the test material was sieved through a 0.5 mm sieve (***Table*** *3.1*). Moreover, on filters with material corresponding to lower size categories (0.250, 0.125 and the bottom, that can be seen in the ***Fig.*** *4.1*), microplastics were found larger than the mesh size, i.e. a larger material, however, has the ability to pass through a finer sieve - this specificity is especially true for fibers and lines. In this connection, the preliminary granulometric separation loses its meaning, while contributing to the complication of the procedure as a whole, as well as additional losses of material and a possible violation of its structure. Accordingly, granulometric separation should be applied either at the sampling site or in laboratory to screen out large particles (usually a 3-5 mm sieve) or use tweezers to completely eliminate screening. Or the use of granulometric separation for sand of a larger fraction to conduct preliminary granulometry on a test sample (at the sampling site) and to determine the size of the granules and their percentage. And the, based on this result, it could be possible determine the feasibility of further use of granulometric separation.

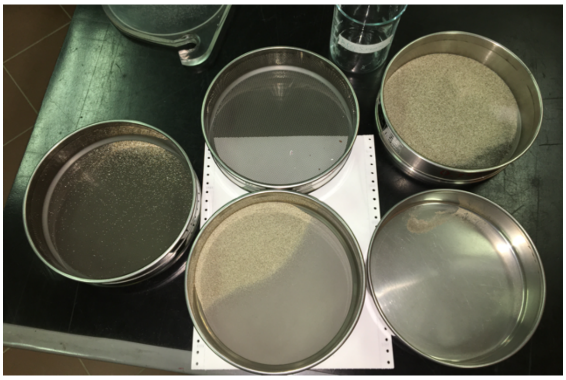


***Fig. 4.1****.Quantity of different class sizes as the result of granulometric separation of sand sample*

However, in this case, the identification of the amount of material more than 0.5 mm played an important role in repeating the experiment of Enders et al (2020) and subsequent detection of the effectiveness of the use of an electrostatic separator, which could not be done due to the almost complete absence of the material of the designated category. Since the efficiency of electrical separation decreases with decreasing material size, particle size separation below 0.5 mm was not used. After electrostatic separation of a part of the sample, 84.04% of the material was eliminated, while the loss was 0.18% after two repetitions. This demonstrates the significant efficiency of this type of separation at low losses if the goal is to reduce the volume of the sample after bulk sampling of the material.

Thus, in the case of fine sand, separation only due to granulometric separation and / or an electric separator is impossible, since material smaller than 500 μm may contain microplastic particles invisible to the naked eye, and organic matter and the sand itself will interfere with identification even under a microscope. Accordingly, in this case, additional densimetric separation is necessary.

The density of the applied NaCl solution is lower than some types of polymers, due to which the efficiency of densimetric separation is reduced. To quantify this efficiency, an additional analysis of the material that has settled to the bottom of the baker (together with sand) should be carried out.

### ➢ *UV Microscopy*

A total of 5 filters were analyzed under a UV microscope. ***Table*** *4.1* shows a comparative characteristic of the number of MPs/g detected and counted as a result of using the UV microscope.

***Table 4.1****. Microplastics abundancy after UV microscope identification*

|  | **after Electrostatic Separation, MPs/g** | **after Densimetric Separation, MPs/g** |  |  |  |
|---|---|---|---|---|---|
| **Dimension** | **unique** | **0.5+1 mm** | **0.250 mm** | **0.125mm** | **bottom** |
| **Closer to the sea** | 3.384 | 56.134 | 2.425 | 3.1 | 383.333 |
| **Further to the sea (~24 m)** | lost | - | - | 4.275 | - |

As can be seen, filters obtained by filtering a larger amount of sand material (for example, 40 grams) have comparable quantitative results, for example 2.4 and 3.1 MPs/g, which is very different from the values obtained by filtering a small amount of material (from 0.2 to 2.5 grams) obtained as a result of granulometric separation. In the second case, the values of 56.1 and 383.3 MPs/g can sometimes be explained by a higher concentration, both with a decrease in the size of sand particles and with their increase (Alomar et al., 2016; Vermeiren et al., 2021). Nevertheless, the comparison of the results obtained from significantly different volumes as MPs/g is incorrect.

While the filter obtained as a result of electrostatic and then densimetric separation differs little from the values obtained as a result of filtering a comparable amount of material, i.e. 3.39 MPs/g (from 31.9 grams of sand) and 2.4 and 3.1 MPs/g (from 40 grams of sand), respectively. This indicates a high-quality separation and a comparable concentration of microplastics as a result of the use of an electric separator, without the need for preliminary separation of the material into classes and, accordingly, a decrease in labor costs. These comparisons were made for the material taken near shoreline.

Then, one of the subsamples was selected representing the material taken at the maximum distance from the sea in order to compare the concentration of microplastics both between the same class and between different beach areas. As a result, in the same class (0.125 mm) a higher concentration of microplastics was found in the zone located about 24 m from the sea - 4.27 MPs/g, compared to 3.1 MPs/g (zone near the seashore). Thus, the far zone is more polluted.

| FILTER | MP Type | Abundance by type | SIZE CATEGORY, mm | | | LIGHT SOURCE | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.1 - 0.49 | 0.5 - 0.99 | 1 - 5 | under UV light | | under LED light | | | | | | | | | | | | | | | |
| | | | | | | FL | NO FL | trasparent | white | blue | black | red | beige | giallo/giallastro | amber | orange | pink | green | gray | multicolor | turquoise | brown | Violet |
| **A(2)_0.5+1 mm** | | | | | | | | | | | | | | | | | | | | | | | |
| | Fiber FB | 8 | | | 8 | 5 | 3 | 3 | | | 2 | | 1 | | | | 1 | | | | 1 | | |
| | Microfiber MFB | 41 | 32 | 9 | | 39 | 2 | 21 | | 2 | | | 12 | | 3 | | 1 | | 2 | | | | |
| | Lines LN | | | | | | | | | | | | | | | | | | | | | | |
| | Microlines ML | 42 | 38 | 4 | | 42 | | 12 | | 2 | | | 26 | 1 | | | 1 | | | | | | |
| | Film FL | | | | | | | | | | | | | | | | | | | | | | |
| | Microfilm MFL | 29 | 29 | | | 29 | | 24 | | | 1 | | 4 | | | | | | | | | | |
| | Pellet PT | | | | | | | | | | | | | | | | | | | | | | |
| | Microbeads MBD | | | | | | | | | | | | | | | | | | | | | | |
| | Fragments FR | | | | | | | | | | | | | | | | | | | | | | |
| | Microfragments MFR | 28 | 28 | | | 27 | 1 | 14 | 4 | 1 | | | 4 | 1 | 2 | | 1 | | | 1 | | | |
| | Foam FM | | | | | | | | | | | | | | | | | | | | | | |
| | Microfoam MFM | | | | | | | | | | | | | | | | | | | | | | |
| | Flacke | | | | | | | | | | | | | | | | | | | | | | |
| | Microflacke | 3 | 3 | | | 3 | | 1 | | | 1 | | 1 | | | | | | | | | | |
| ***Quantity TOT*** | | ***151*** | **130** | **13** | **8** | **145** | **6** | **75** | **4** | **5** | **4** | | **48** | **2** | **5** | | **4** | | **2** | **1** | **1** | | |
| **A(2)_0.250 mm** | | | | | | | | | | | | | | | | | | | | | | | |
| | Fiber FB | 6 | | | 6 | 5 | 1 | 2 | | | 2 | 1 | | | | | 1 | | | | | | |
| | Microfiber MFB | 24 | 17 | 7 | | 22 | 2 | 11 | | 2 | 4 | | 4 | | | 1 | 1 | | | 1 | | | |
| | Lines LN | 2 | | | 2 | 2 | | | | | | | 2 | | | | | | | | | | |
| | Microlines ML | 20 | 20 | | | 20 | | 6 | | | | | 14 | | | | | | | | | | |
| | Film FL | | | | | | | | | | | | | | | | | | | | | | |
| | Microfilm MFL | 22 | 22 | | | 20 | 2 | 15 | | | 2 | | 5 | | | | | | | | | | |
| | Pellet PT | | | | | | | | | | | | | | | | | | | | | | |
| | Microbeads MBD | 3 | 3 | | | 1 | 2 | | | | | | | 3 | | | | | | | | | |
| | Fragments FR | | | | | | | | | | | | | | | | | | | | | | |
| | Microfragments MFR | 11 | 11 | | | 10 | 1 | 5 | 1 | | 1 | | 3 | | 1 | | | | | | | | |
| | Foam FM | | | | | | | | | | | | | | | | | | | | | | |
| | Microfoam MFM | 1 | | 1 | | 1 | | | | | | | | | 1 | | | | | | | | |
| | Flacke | | | | | | | | | | | | | | | | | | | | | | |
| | Microflacke | 8 | 8 | | | 6 | 2 | 3 | 1 | | 2 | | 1 | | | | 1 | | | | | | |
| ***Quantity TOT*** | | ***97*** | **81** | **8** | **8** | **87** | **10** | **42** | **2** | **2** | **11** | **1** | **29** | **3** | **2** | **1** | **3** | | | **1** | | | |

***Table 4.2***. *Detailed quantitative analysis of microplastic particles (by type, color and size) found in A (2)_0.5+1 mm and A (2)_0.250 mm subsamples*

| FILTER | MP Type | Abundance by type | SIZE CATEGORY, mm | | | LIGHT SOURCE | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.1 - 0.49 | 0.5 - 0.99 | 1 - 5 | under UV light | | under LED light | | | | | | | | | | | | | | | |
| | | | | | | FL | NO FL | trasparent | white | blue | black | red | beige | giallo/giallastro | amber | orange | pink | green | gray | multicolor | turquoise | brown | Violet |
| **A(2)_0.125 mm** | | | | | | | | | | | | | | | | | | | | | | | |
| | Fiber FB | 6 | | | 6 | 6 | | 4 | | 1 | | | | | 1 | | | | | | | | |
| | Microfiber MFB | 39 | 33 | 6 | | 35 | 4 | 18 | | 1 | 2 | 2 | 11 | 3 | | | 1 | | 1 | | | | 1 |
| | Lines LN | | | | | | | | | | | | | | | | | | | | | | |
| | Microlines ML | 26 | 25 | 1 | | 26 | | 14 | | | | | 12 | | | | | | | | | | |
| | Film FL | | | | | | | | | | | | | | | | | | | | | | |
| | Microfilm MFL | 21 | 21 | | | 18 | 3 | 13 | | | 1 | | 5 | 1 | | | | | | 1 | | | |
| | Pellet PT | | | | | | | | | | | | | | | | | | | | | | |
| | Microbeads MBD | 3 | 3 | | | | 3 | | | | 1 | | 1 | 1 | | | | | | | | | |
| | Fragments FR | | | | | | | | | | | | | | | | | | | | | | |
| | Microfragments MFR | 21 | 20 | 1 | | 16 | **4** | 6 | 1 | | 1 | | 6 | 2 | 1 | | 1 | | 2 | | | 1 | |
| | Foam FM | | | | | | | | | | | | | | | | | | | | | | |
| | Microfoam MFM | | | | | | | | | | | | | | | | | | | | | | |
| | Flacke | | | | | | | | | | | | | | | | | | | | | | |
| | Microflacke | 8 | 6 | 2 | | 3 | | 2 | | | 2 | | 3 | 1 | | | | | | | | | |
| ***Quantity TOT*** | | ***124*** | **108** | **10** | **6** | **110** | **14** | **57** | **1** | **2** | **7** | **2** | **38** | **8** | **2** | | **2** | | **3** | | | **1** | **1** |
| | | | | | | | | | | | | | | | | | | | | | | | |
| **A(2)_fondo** | | | | | | | | | | | | | | | | | | | | | | | |
| | Fiber FB | 4 | | | 3 | 4 | | 1 | | | | | | | | | 2 | | | | | | |
| | Microfiber MFB | 33 | 24 | 9 | | 29 | 4 | 16 | | 8 | 2 | 1 | 2 | | | | 3 | 1 | | | | | |
| | Lines LN | | | | | | | | | | | | | | | | | | | | | | |
| | Microlines ML | 18 | 18 | 1 | | 18 | | 7 | | 1 | | | 11 | | | | | | | | | | |
| | Film FL | | | | | | | | | | | | | | | | | | | | | | |
| | Microfilm MFL | 20 | 20 | | | 19 | 1 | 12 | | | 2 | | 5 | | | | | 1 | | | | | |
| | Pellet PT | | | | | | | | | | | | | | | | | | | | | | |
| | Microbeads MBD | | | | | | | | | | | | | | | | | | | | | | |
| | Fragments FR | | | | | | | | | | | | | | | | | | | | | | |
| | Microfragments MFR | 11 | 9 | 2 | | 11 | | 3 | 1 | | | | 4 | | 1 | | | 1 | | 1 | | | |
| | Foam FM | | | | | | | | | | | | | | | | | | | | | | |
| | Microfoam MFM | 4 | 3 | 1 | | 3 | 1 | | 3 | | | | | 1 | | | | | | | | | |
| | Flacke | | | | | | | | | | | | | | | | | | | | | | |
| | Microflacke | 2 | 2 | | | 1 | 1 | | | | 2 | | | | | | | | | | | | |
| ***Quantity TOT*** | | ***92*** | **76** | **13** | **3** | **85** | **7** | **39** | **4** | **9** | **6** | **1** | **22** | **1** | **1** | | **5** | **3** | | **1** | | | |

***Table 4.3***. *Detailed quantitative analysis of microplastic particles (by type, color and size) found in A (2)_0.125 mm and A (2)_bottom subsamples*

| FILTER | MP Type | Abundance by type | SIZE CATEGORY, mm | | | LIGHT SOURCE | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.1 - 0.49 | 0.5 - 0.99 | 1 - 5 | under UV light | | under LED light | | | | | | | | | | | | | | |
| | | | | | | FL | NO FL | trasparent | white | blue | black | red | beige | giallo/giallastro | amber | orange | pink | green | gray | multicolor | turquoise | brown | Violet |
| **C(2)_0.125** | | | | | | | | | | | | | | | | | | | | | | | |
| | Fiber FB | 6 | | | 6 | 6 | | 3 | | 2 | | 1 | | | | | | | | | | | |
| | Microfiber MFB | 38 | 33 | 5 | | 30 | 8 | 16 | | 10 | 5 | 1 | 2 | | | | 1 | 2 | 1 | | | | |
| | Lines LN | | | | | | | | | | | | | | | | | | | | | | |
| | Microlines ML | 36 | 35 | 1 | | 36 | | 31 | | | | | 5 | | | | | | | | | | |
| | Film FL | | | | | | | | | | | | | | | | | | | | | | |
| | Microfilm MFL | 27 | 26 | 1 | | 25 | 2 | 14 | 1 | 1 | 2 | | 5 | | | | 2 | 1 | | | | 1 | |
| | Pellet PT | | | | | | | | | | | | | | | | | | | | | | |
| | Microbeads MBD | 4 | 4 | | | 4 | | | | | | | | 4 | | | | | | | | | |
| | Fragments FR | 1 | | | 1 | 1 | | | | | | | | 1 | | | | | | | | | |
| | Microfragments MFR | 51 | 51 | | | 50 | **1** | 16 | 2 | 1 | 4 | | 13 | 3 | 6 | | | 2 | | 1 | | 3 | |
| | Foam FM | | | | | | | | | | | | | | | | | | | | | | |
| | Microfoam MFM | 2 | 2 | | | 2 | | 1 | 1 | | | | | | | | | | | | | | |
| | Flacke | | | | | | | | | | | | | | | | | | | | | | |
| | Microflacke | 6 | 5 | 1 | | 5 | **1** | | | | 1 | | 3 | | 1 | | | | 1 | | | | |
| ***Quantity TOT*** | | ***171*** | **156** | **8** | **7** | **159** | **12** | **81** | **4** | **14** | **12** | **2** | **28** | **8** | **7** | | **3** | **5** | **2** | **1** | | **4** | |
| | | | | | | | | | | | | | | | | | | | | | | | |
| **A(1)_SE** | | | | | | | | | | | | | | | | | | | | | | | |
| | Fiber FB | 2 | | | 2 | 1 | 1 | 2 | | | | | | | | | | | | | | | |
| | Microfiber MFB | 26 | 19 | 7 | | 26 | | 15 | | 1 | 1 | | 6 | | 1 | | | | 1 | | | | 1 |
| | Lines LN | 2 | | | 2 | 2 | | | | | | | 2 | | | | | | | | | | |
| | Microlines ML | 22 | 22 | | | 22 | | 13 | | | | | 8 | | | | | | 1 | | | | |
| | Film FL | | | | | | | | | | | | | | | | | | | | | | |
| | Microfilm MFL | 19 | 18 | 1 | | 17 | 2 | 13 | | | | | 5 | 1 | | | | | | | | | |
| | Pellet PT | | | | | | | | | | | | | | | | | | | | | | |
| | Microbeads MBD | | | | | | | | | | | | | | | | | | | | | | |
| | Fragments FR | | | | | | | | | | | | | | | | | | | | | | |
| | Microfragments MFR | 30 | 30 | | | 28 | **2** | 7 | | 1 | 3 | | 7 | 4 | 2 | | | 1 | | 1 | | 1 | |
| | Foam FM | | | | | | | | 3 | | | | | | | | | | | | | | |
| | Microfoam MFM | | | | | | | | | | | | | | | | | | | | | | |
| | Flacke | | | | | | | | | | | | | | | | | | | | | | |
| | Microflacke | 7 | 7 | | | 6 | **1** | 2 | | | 2 | | 2 | | | | | | | | | 1 | |
| ***Quantity TOT*** | | ***108*** | **96** | **8** | **4** | **102** | **6** | **52** | **3** | **2** | **6** | | **30** | **5** | **3** | | | **1** | **2** | **1** | | **2** | **1** |

***Table 4.4.*** *Detailed quantitative analysis of microplastic particles (by type, color and size) found in C (2)_0.125 mm and A (1)_Electrostatic Separation subsamples*

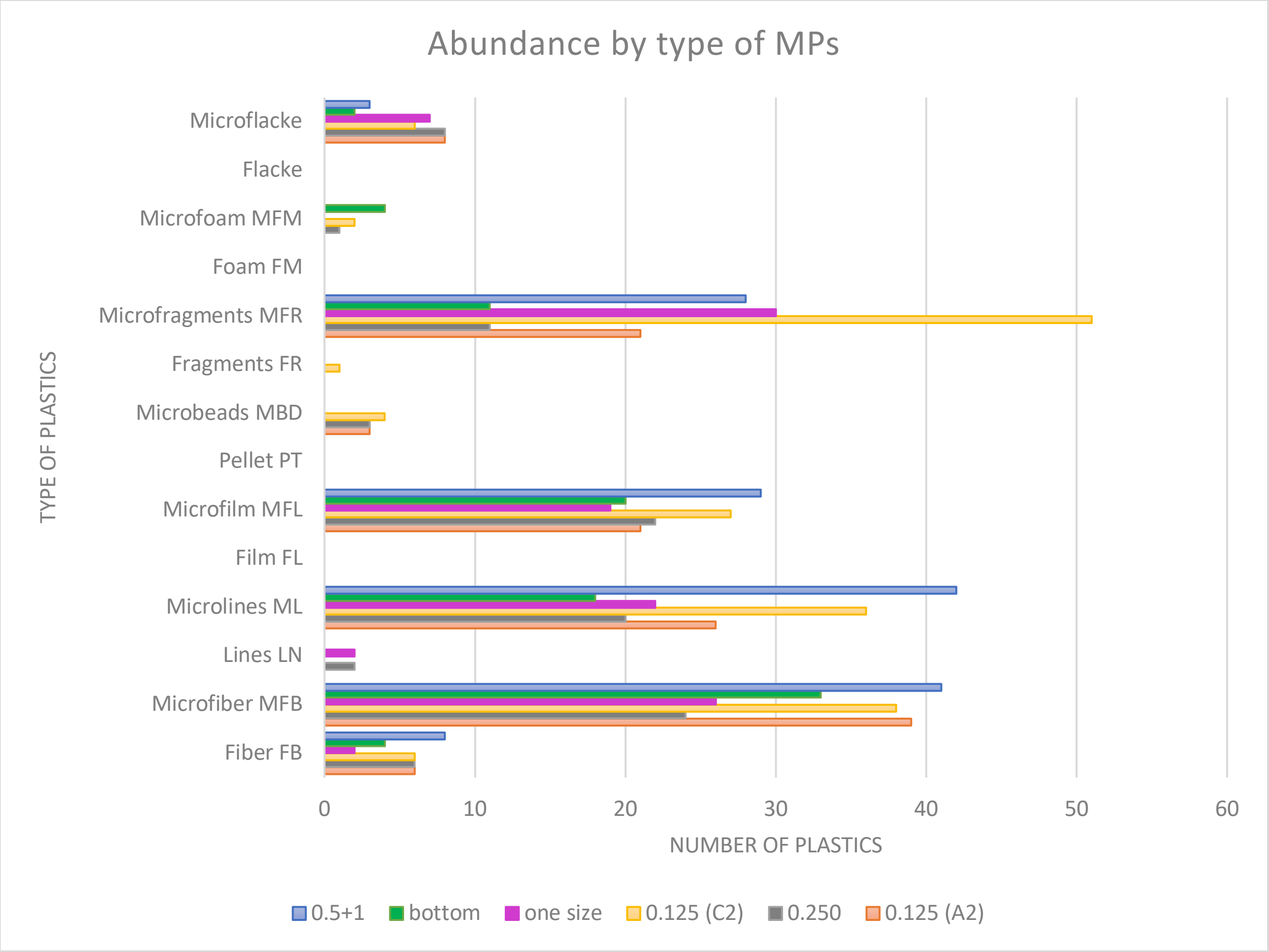


***Fig. 4.2.**. MPs quantity in relation to the type and subsample*

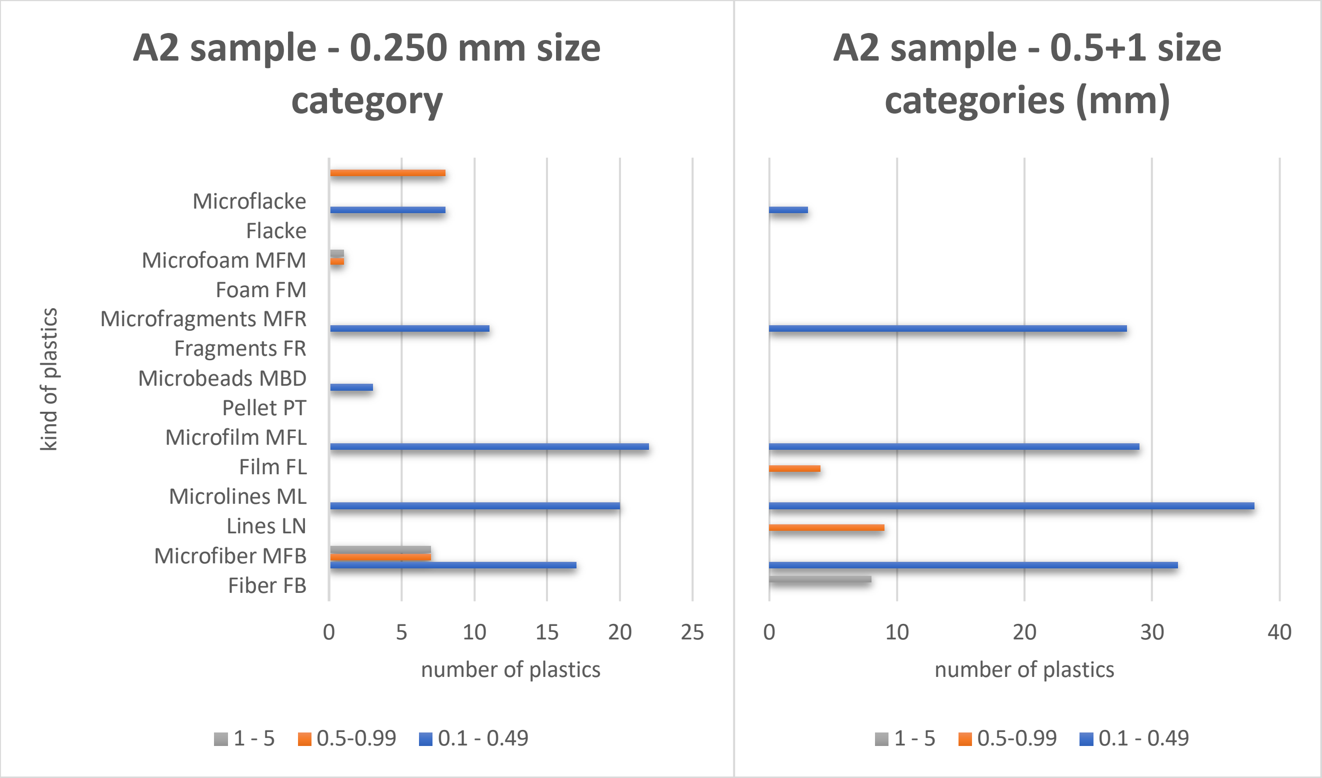


***Fig. 4.3.** MPs quantity in relation to the type and size category of A2_0.250mm (left) and 0.5+1mm subsamples*

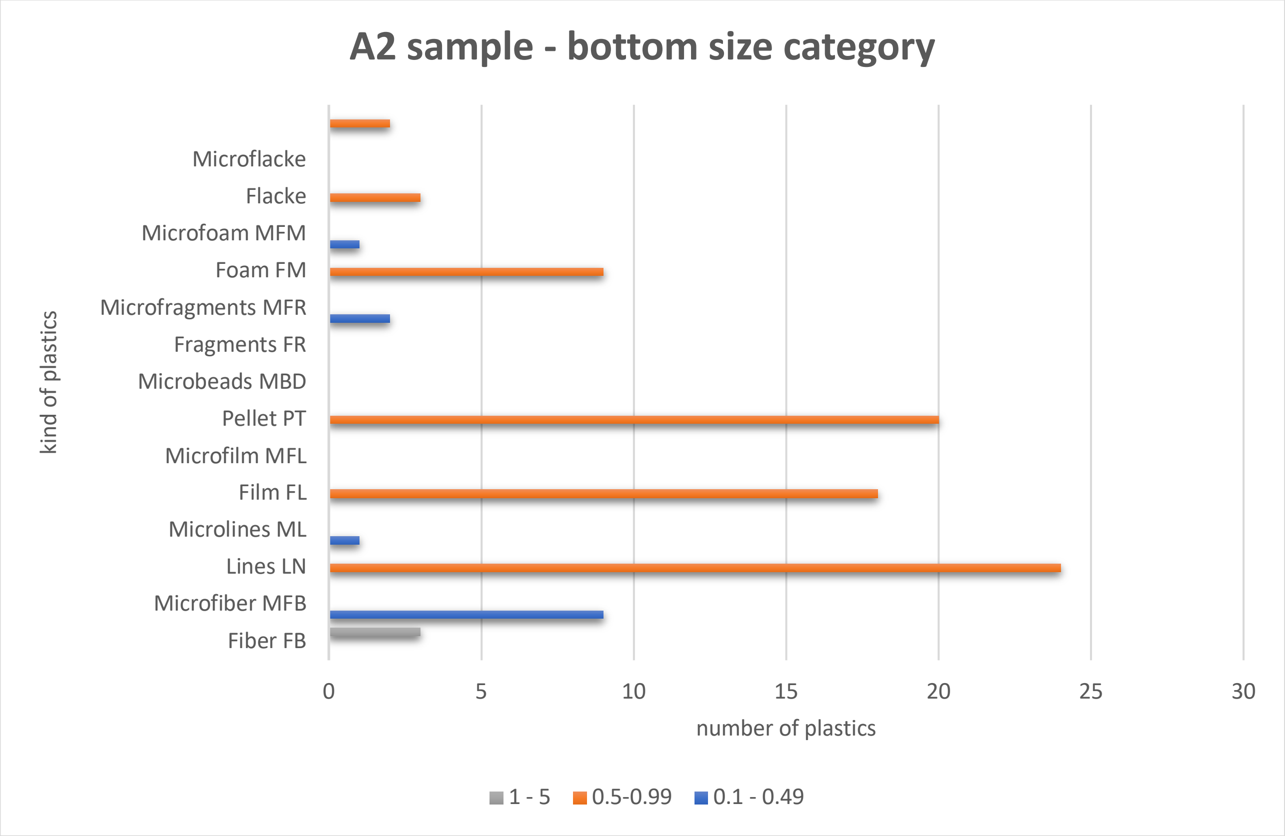


***Fig. 4.4.*** *MPs quantity in relation to the type and size category of A2_bottom subsample*

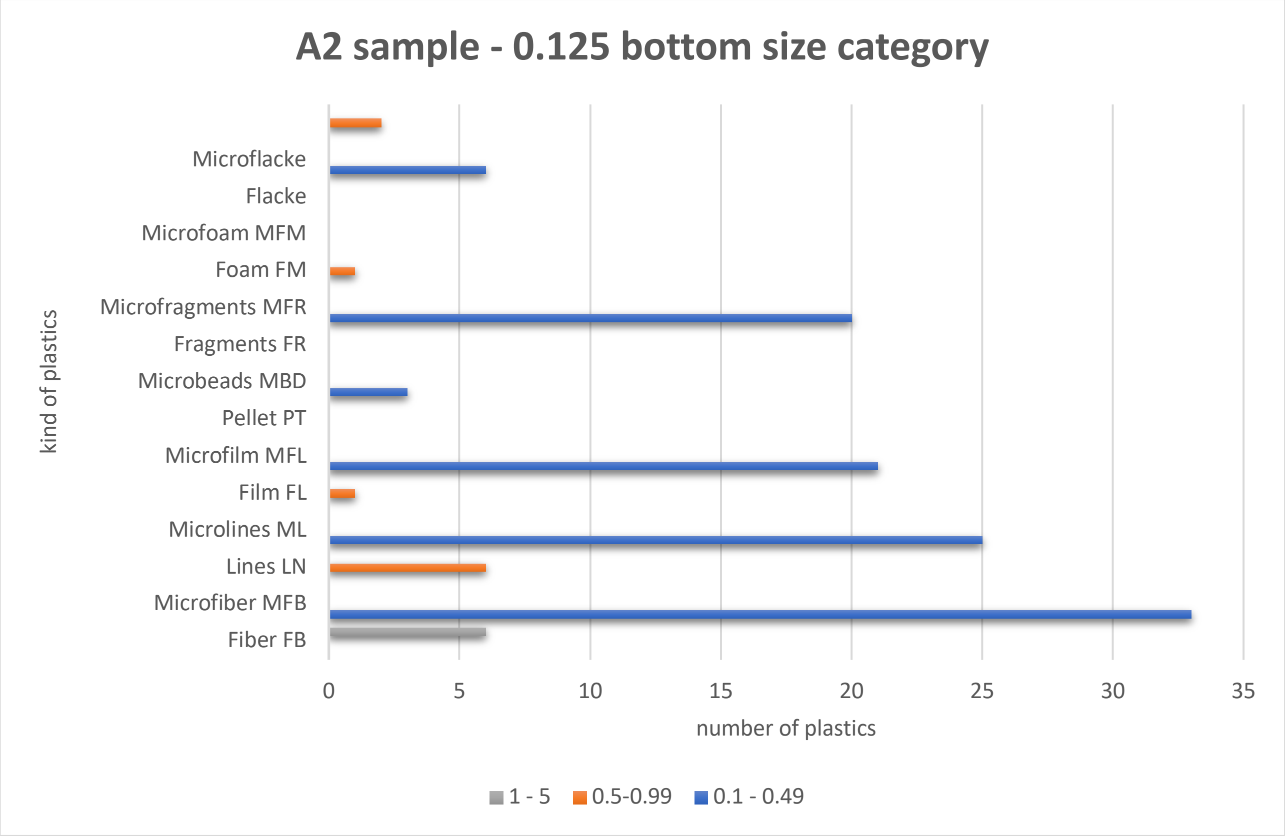


***Fig. 4.5****. MPs quantity in relation to the type and size category of A2_0.125mm subsample*

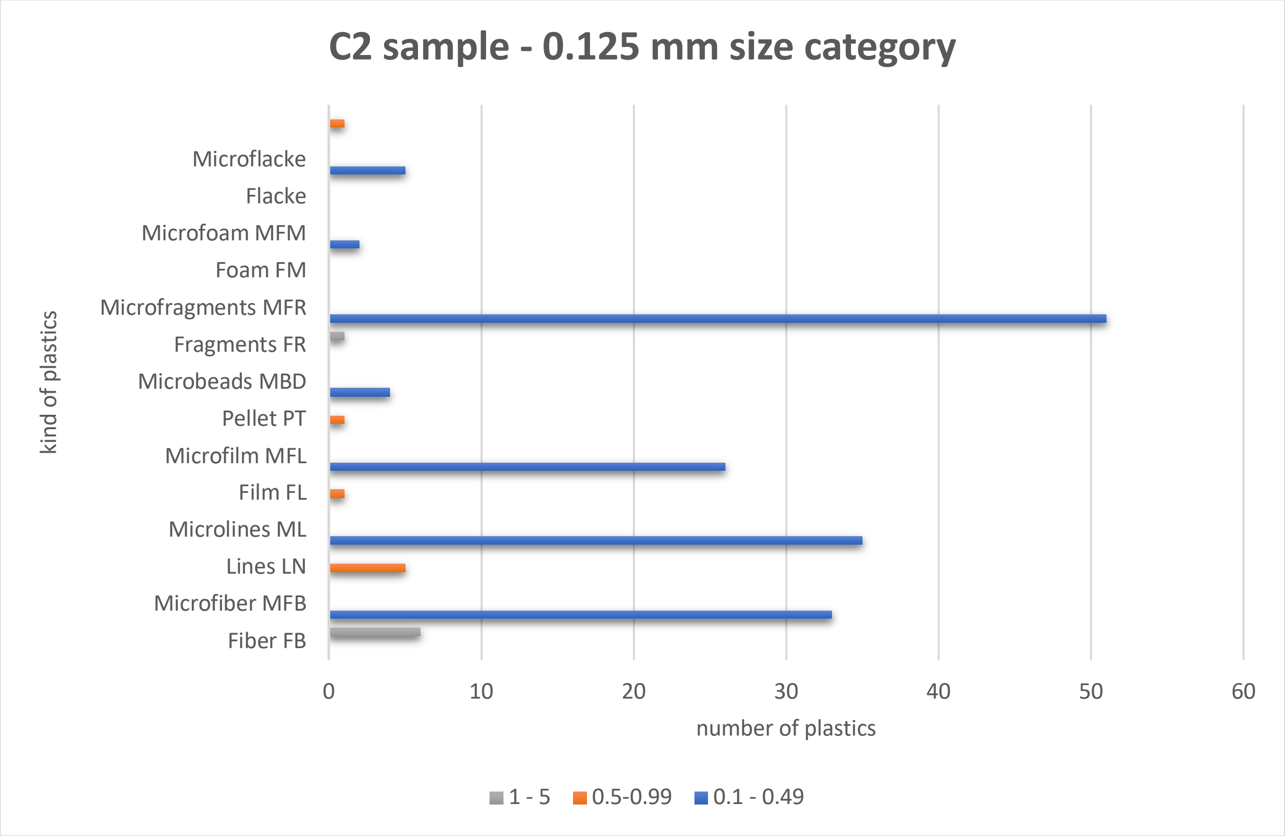


*Fig. 4.6. MPs quantity in relation to the type and size category of C2_ 0.125mm subsample*

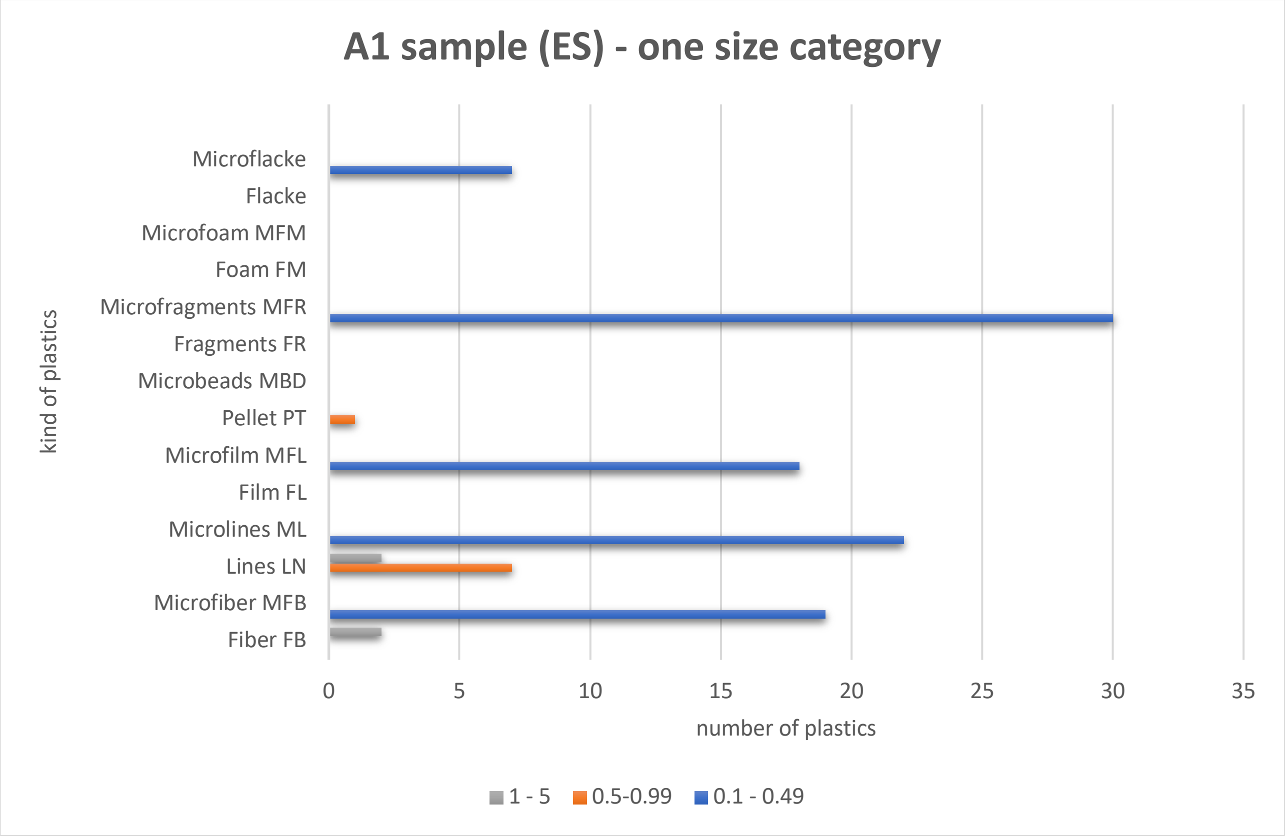


*Fig. 4.7. MPs quantity in relation to the type and size category of A1_ 0.125mm subsample*

A detailed quantitative and qualitative analysis for each of the five filters is given in ***Tables*** *4.2, 4.3, 4.4* and also some results can be seen in comparative graphics in ***Fig.*** *4.2, 4.3, 4.5, 4.6, 4.7*. According to which the most common type of microplastic, in most cases, is a fluorescent fiber as well as lines of a transparent color (31.36 and 22.61% of the total number of microplastic particles found n=743) and a size category up to 0.5mm. The second most popular color is beige, which is best combined with similar colors such as yellowish and yellow as they are problematic to distinguish from each other; especially since plastic, especially transparent, characteristically changes under the influence of ultraviolet radiation, as well as a result of the digestive processes of the biota (Khan et al., 2018; Cole et al., 2014), acquiring a shade of varying degrees of yellowness, which explains the dominance of this colors along with transparent. Both of these colors are easy to miss in visual identification as yellowish tints are often found in natural materials (organics, minerals) which are just as often fluorescent (Bertoldi et al., 2021). Therefore, in such cases, the level of error can be the highest, especially considering the greatest presence of translucent and yellowish microplastics, as well as their size category (less than 0.5 mm), in which the level of identification error can reach up to 70%.

However, the filter obtained as a result of electrostatic separation, as well as the filter representing the remote zone of the beach (***Fig***. *4.8*), differed markedly from the others, since on them was found a prevailing number of "fragments" than other forms - 27.78 and 29.82%, respectively. Also, a characteristic difference of the A(1)_SE filter is the presence of a visually larger amount of material less than 0.1 mm, among which a large number (and more than on other filters) of nanobeads of presumably synthetic origin is clearly visible (***Fig***. *4.12*). But in general, can be noted the almost equal dispersion among different forms found on the filters (***Fig.*** *4.2*), which explains the rather low percentages of dominance of one form over another in the case of microplastics.

Also on all filters, in a huge amount (about 1000 or more items) the fibers were found. Their visual characteristics corresponded more to the organic nature (for example, cotton). Because of their abundancy it was difficult to visually identify and count microplastics; as well as various organic particles were not completely removed after applying the $H_2O_2$ solution due to its insufficient concentration (15%) for this type of material (sand samples) that had a large presence of different natural and organic materials (***Fig.*** *4.9*). Thus, the study provided by *Sturm et al. (2021)* in relation to the substantial decrease or elimination of the organic matter fluorescent signal was not confirmed in this study, which negatively affects the detection and recognition of microplastics. This demonstrates the impossibility of using an universal protocol for preparing samples from different environments, as well as the need to use solutions with a stronger concentration, for example, 30-35% $H_2O_2$, which has shown its effectiveness in many scientific studies (Nuelle et al, 2014; Vermeiren et al . 2020), despite the possible slight damage to the plastic as a result of its use. However, in this work, a green filter (green fluorescence), which has been repeatedly shown to be effective, was not used in order to achieve similar results demonstrated by *Sturm et al*. which, perhaps, to some extent could neutralize the presence of organic matter and improve the identification process.

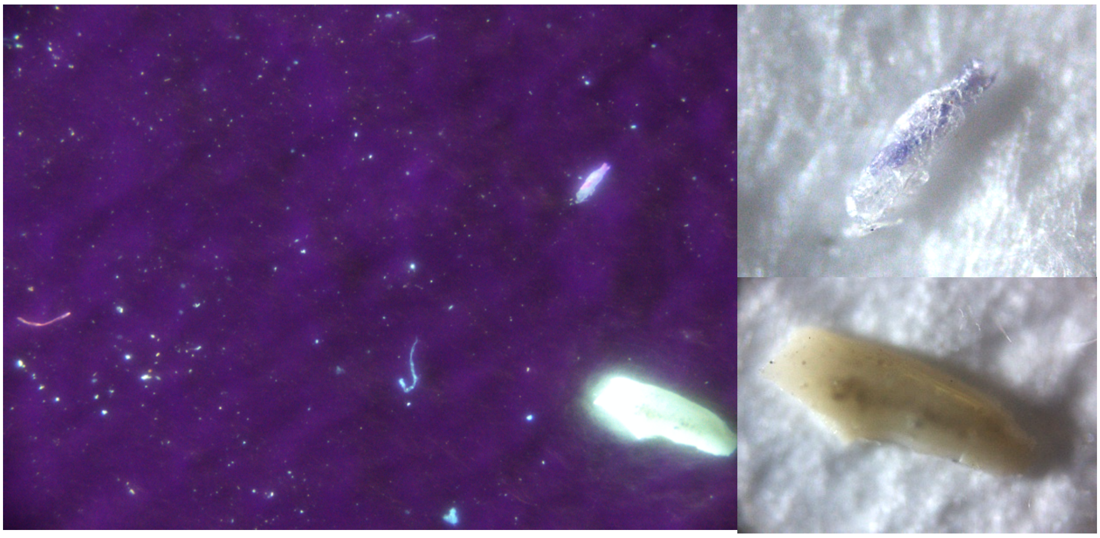


***Fig**. 4.8.Microplastic fragments from the sample taken at a distance of 24 meters from the sea (2.5x magnification – left; 10x – right)*

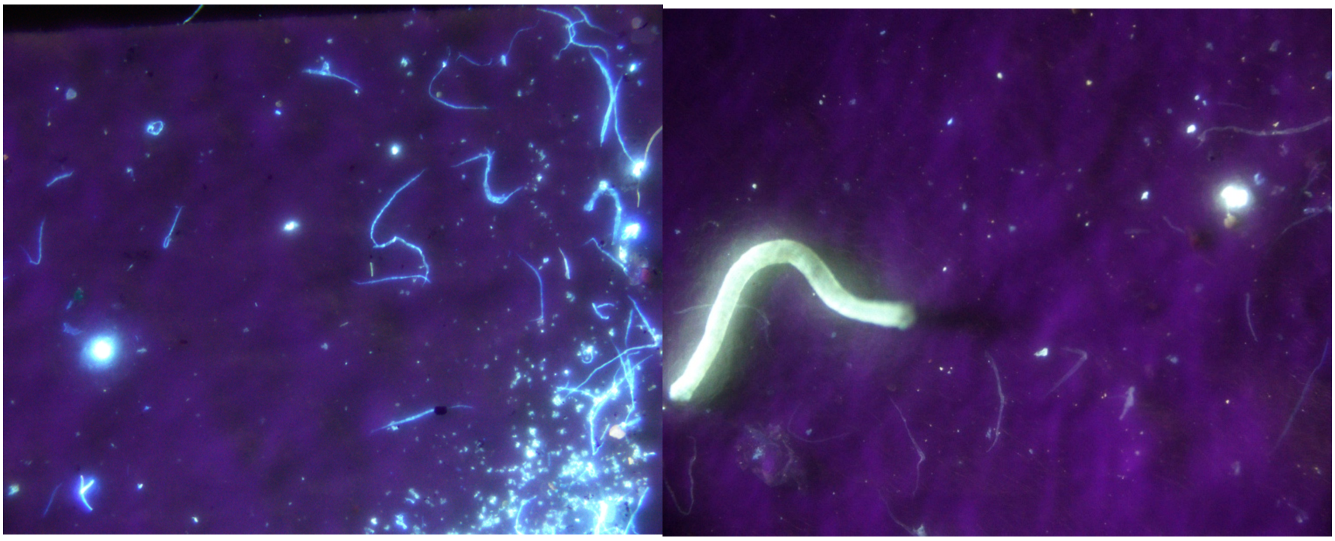


***Fig**. 4.9. Prevailing abundance of natural and organic fluorescent materials (2.5x magnification)*

Among the interesting features of various types and forms of microplastics, revealed as a result of visual identification using a microscope with UV, the following can be indicated:

- detection of material and microplastics with a size exceeding its category, which indicates the low efficiency and uselessness of granulometric separation;

- the prevalence of fluorescent material (89.91%) over non-fluorescent, both among microplastics and among organic and inorganic materials, which confirms the thesis that fluorescence itself cannot be an identification method, but can contribute to it because there is an obvious correlation between the amount of microplastics found and its fluorescent properties. Whereas *Norén et al, 2007* advise using fluorescence microscopy in combination with magnification in the case of white or transparent particles/elements to rule out their organic origin, this is not a very reliable method since many organic particles are also fluorescent (***Fig**.4.9*).

- microplastics that did not have fluorescent properties almost always had a black or blue color, which means that they were clearly visible under simple LED lighting - this result confirms numerous studies that have revealed a similar trend. Moreover, examples of black and blue microplastics were found that exhibited fluorescence (more or less intense) in places of partial or complete loss of color (***Fig***. *4.10*). Also, some types of microplastics, mainly fibers, had several colors at the same time, and therefore were listed in the "multicolor" category. Which, in turn, refutes one of the rules of *Noren et al.*, o "homogeneously colored particles", since neither the color itself nor its more or less harmonious distribution helps in identifying microplastics. many fibers of uniform bright colors were found, apparently of non-synthetic or semi-synthetic origin; and also this rule does not take into account the predisposition of plastics to abrasion, deformation, destruction and loss of color in the environment.

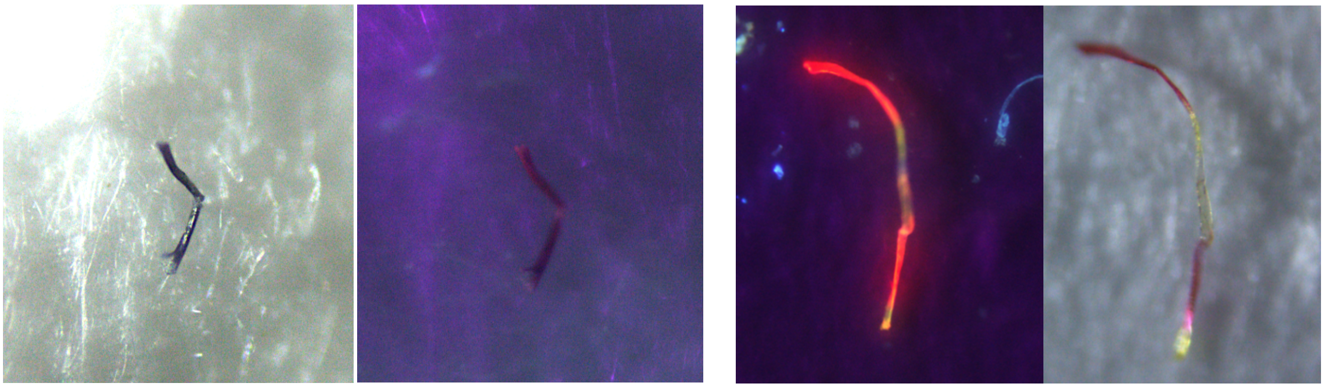


***Fig. 4.10***. *Synthetic microfibers of black and multi colors with and without UV light (10x magnification)*

- it was noted that fibers with a thicker cross section (not flat) are likely to be synthetic (***Fig***. *4.11*). This feature can be observed as in the examples of fibers taken for the preliminary test with destruction (***Fig***. *3.9-3.12; 3.17*), where a cotton fiber was flat and more twisted around its axis (initially) while polyester was more straight (before destruction) and had a thicker diameter. Also, based on a review of microlabgallery.com/gallery-fiber.aspx photos, it can be concluded that this observation and trend are correct. However, this can be confirmed as long as the fiber is not severely destroyed, after which it becomes difficult or almost impossible to distinguish from natural one.

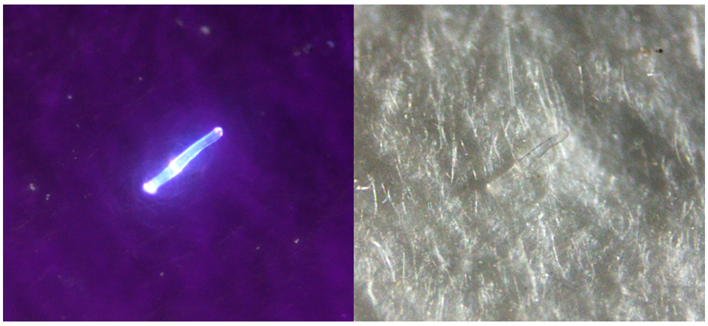


***Fig***. ***4.11***. *Synthetic microfiber (10x magnification)*

- microbeads or nanobeads, despite their small size (less than 0.1 mm i.e. nanoplastics 0.6-0.8 mm in size), are more easily identified than even larger fibers, fragments, film, due to their characteristic shape, color and, in general, due to a sharper image produced by a microscope camera, at a magnification of 10x (***Fig***. *4.12*). This paradox has also been noted by other researchers (Bertolidi et al., 2021; Ehlers, Sonja & Manz, W et al, 2019). However, due to the indicated identification threshold of 0.1 mm, this type of nanoplastic was not taken into account in the calculations;

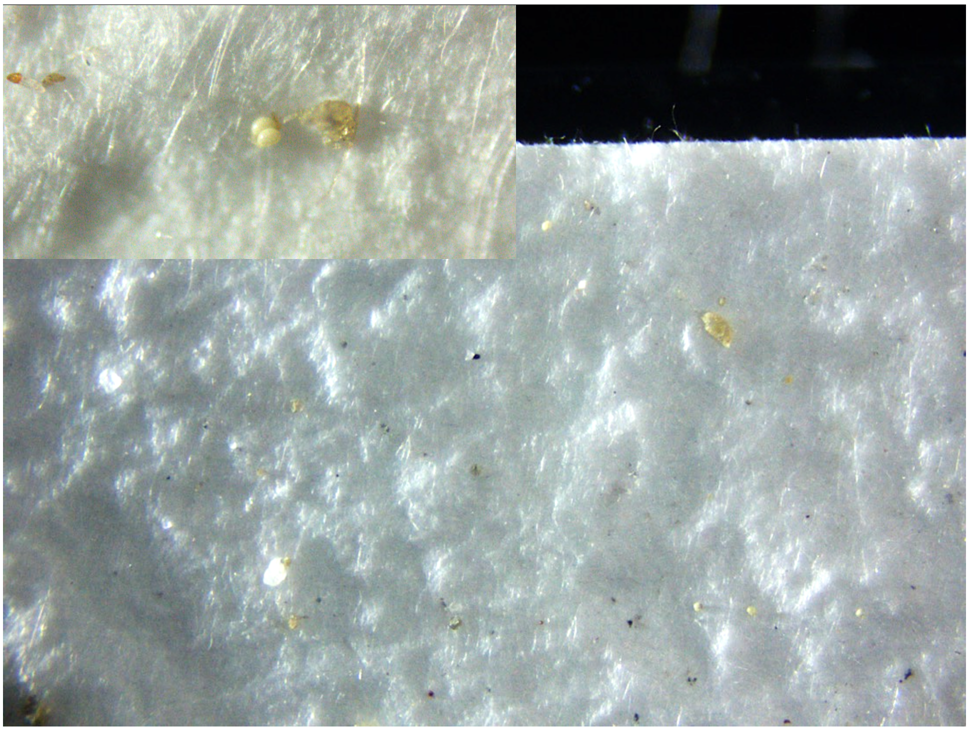
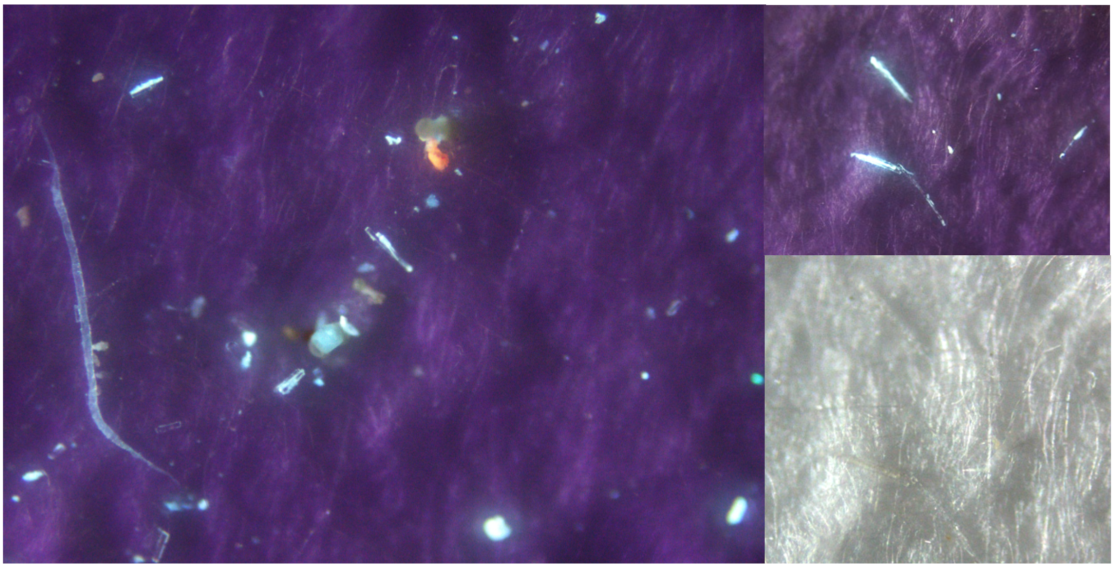


***Fig. 4.12***. *Nanobeads (size below 0.1mm) abundance*

- the most common particle size in all subsamples was within the range of 0.1 to 0.5 mm (***Fig.*** *4.3-4.7*), which correlates with the results of other studies;

- typical examples of the additional categories "microlines" and "microflackes" adopted in this work are shown in ***Fig***. *4.13.* With regard to microlines, a straight, rigid (unlike fibers) shape can be noted. It breaks noticeably easily into smaller pieces of microplastic when interacting with the environment and thus moving from the category of micro to nanoplastics. Therefore, many similar microlines were found, as well as nanolines, absolutely on all filters and in all categories, but especially a lot in the subsample selected from the far line of the beach (~ 24m from the seashore). Whereas microflackes were almost underrepresented. As can be seen, this category included particles of microplastics that looked like flakes, having some thickness (unlike microfilm) but not having more or less clear shapes or visually not looking solid (like microfragments).



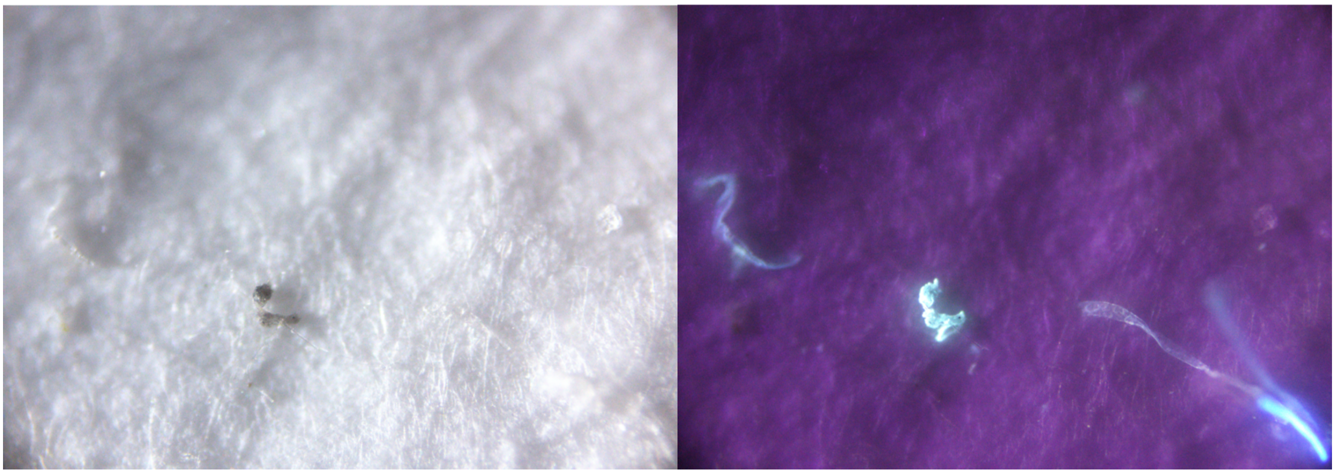


***Fig. 4.13***. *Microlines (above) and microflackes (below) plastic particles under LED and UV light (10x magnification)*

### ➢ *Nile red*

Nile Red staining revealed n=127 (*A(2)_1+0.5mm*) and n=131 (*A(2)_fondo*) items of microplastic particles found in 2.69 and 0.24 grams of sand, respectively. All colored particles, as well as their edges only, were counted and taken as microplastics. ***Table*** *4.5* shows the results of identification using Nile Red dye in categories sizes >0.1mm and <0.1mm. All colored, fluorescent and clearly visible particles of all sizes less than 0.1 mm (seen via the UV microscope) were additionally counted as in the study of *Orb Media* by *Mason et al., (2018)*. This additional count was carried out due to the number of micro and nanoplastics (or particles that can be them) in the class from 0.5 mm and below in order to have an idea of their possible number and possible degree of contamination. This proposed hypothesis is confirmed also by the study of *Enri-Cassola et al. (2017)* who used Nile Red dye and determined that the vast majority of nanoplastics obtained from a single sample were in the 40-30 µm categories, and those that acquired a characteristic fluorescence after staining were of synthetic origin, while those that did not stain had non-plastic signatures, which was determined using Raman spectroscopy. However, given the fact that sample preparation procedures, Nile Red concentrations, types of solutions and colors of wavelengths filters differ, it is necessary to take into account that not only micro and nanoplastics can be stained, which means that this result should be taken only as potentially possible and with a possible false-positive staining fraction and not as an actual one. That is why it is desirable to be subjected to additional verifications using more technologically advanced methods (analytical i.e. Raman, FT-IR) for the determination of chemical composition of colored particles.

***Table 4.5***. *Quantity of the particles stained with NR found on two filters and divided in 2 size categories, where the results in the category size less than 0.1 mm are indicative/potential but not actual*

| **FILTER** | **Colored particles TOT quantity, items** | |
|---|---|---|
| | above 0.1 mm | below 0.1 mm |
| **A(2)_0.5+1 mm** | *127* | *962* |
| **A(2)_bottom** | *131* | *859* |

After applying the Nile Red dye in accordance with the above procedure, the results were identified and confirmed in other works, namely:

- the insufficiency of using only UV lighting, but also other filter/s such as green and orange, for more accurate results, as has been shown in numerous studies, since certain types of plastic (for example, PVC and PP) do not stain well, except for damaged ones parts and cut points, and then do not appear under ultraviolet light as colored objects;

- an increase in the concentration or maintaining the current concentration, but also an increase in the reaction time (up to half an hour) after applying the dye to the filter and before sending it to the oven for drying, can also lead to an improvement in the staining result;

- the organic matter present on the filter is colored acquiring a purple hue, but under UV light it practically does not contrast with the background also colored purple.

- the dyed background gives a certain contrast between the microplastics, which tend to turn a more contrasting color from orange to bright red (visible mainly under UV light), while numerous natural fibers do not stain at all even in places of breakage, shearing or damage, as demonstrated in ***Fig.*** *4.14.* The elongated red lines can also be seen which is the remaining dye, that in general does not greatly interfere with visual identification. However, the filter can be additionally washed and this could improve the visual characteristics of some microplastic particles by increasing the contrast.

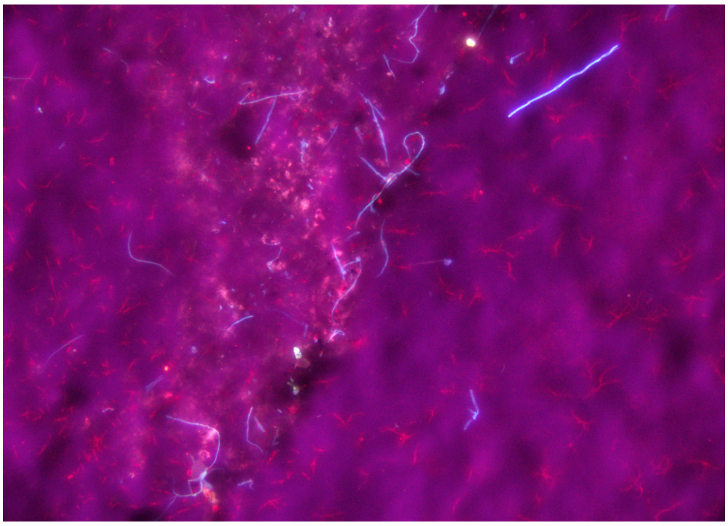


***Fig. 4.14.*** *Colored organic material showing a weak fluorescent signal and pink color (10x magnification)*

### ➢ *Comparison of methods*

Quantitative comparison of the results between filters visually identified only with a UV microscope and stained with Nile Red is presented in ***Table*** *4.6.* According to which the difference between the number of microplastics when using both methods is insignificant, in the case of the filter *A(2)_0.5+1mm*. However, on the same filter, when applying the first or second method, not the same microplastic particles were identified, but different ones in about half of the cases, that is, for example, a fiber that did not fit the classical identification criteria was not taken into account in the calculation as a microplastic, but when applied dye Nile Red was stained (in whole or in part) and then taken into account in the calculation in the second result; and vice versa, in the first case, the particle was defined as a microplastic since. visually met the criteria but then was not painted. However, the inability to stain may depend on: the type of plastic, as has been previously demonstrated in experiment and identified by many scientific articles; from color, since black and blue colors are not able to stain and fluoresce due to their own color; from the staining procedure and methods of visualization of objects subjected to staining.

Therefore, the indicated results may also not reflect a complete picture of the concentration and contamination of microplastics, both in the case of identification using a microscope and using Nile Red dye. And in general, the quantitative comparison between the two methods is conditional, especially for filters obtained as a result of the separation of a small amount of material.

***Table 4.6.*** *Quantitative comparison of microplastic particles detected with only UV microscope and after Nile Red staining*

| | **Under UV Microscope, items** | | | | **Under UV microscope and NileRed, items** | | | |
|---|---|---|---|---|---|---|---|---|
| **Dimension** | **0.5+1 mm** | **0.250 mm** | **0.125mm** | **bottom** | **0.5+1 mm** | **0.250 mm** | **0.125mm** | **bottom** |
| **Closer to the sea** | *151* | | | *92* | *127* | | | *131* |

An example of a synthetic fiber before and after dyeing is shown in ***Fig****. 4.15*:

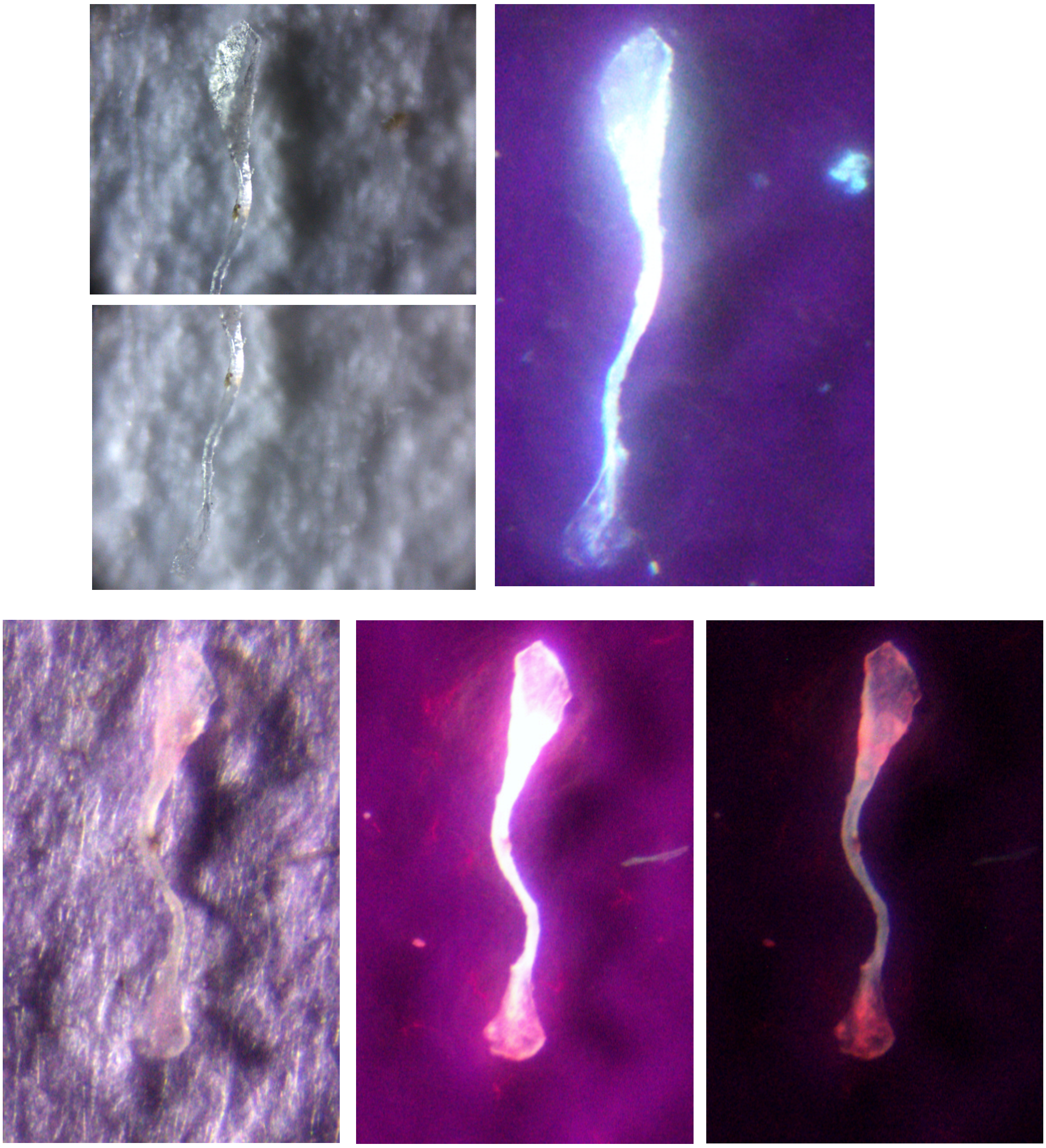


***Fig. 4.15.*** *Synthetic fiber before and after Nile Red staining under UV and LED light (10x and 2.5x magnification)*

As can be seen (***Fig.*** *4.15*), this synthetic transparent fiber was stained precisely in places subjected to degradation as a result of environmental exposure, which confirms the results of a similar experiment on synthetic fibers in the laboratory. Visual identification under UV light and Nile Red both have a certain degree of error. Some artificial but not synthetic fibers can be both fluorescent and colored by Nile Red, so there may be an overestimation of microplastics.

Also, comparing the two methods of identification, it was noticed that some microplastic particles were tinted (from purple to red) and were noticeable even under LED light. While others remained transparent under LED light but glow brightly under UV light. For example, the nanobeads acquired a purple color and a strong red fluorescence (***Fig.*** *4.17*) while before staining they had a yellowish tint and a weak whitish fluorescence (***Fig.*** *4.12*), as did the microlines (***Fig.*** *4.18*), as well as the microfilm (***Fig.*** *4.19*) and microfragment (***Fig.*** *4.16*).

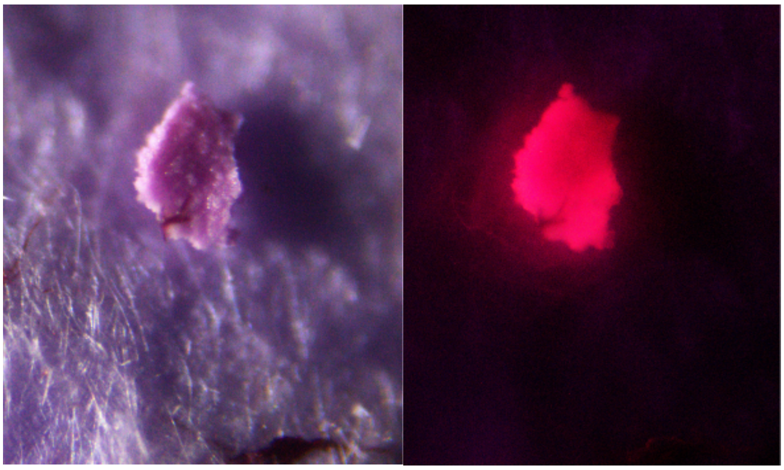


***Fig. 4.16**. Microfragment after Nile Red staining under LED (left) and UV light (right), 10x magnification*

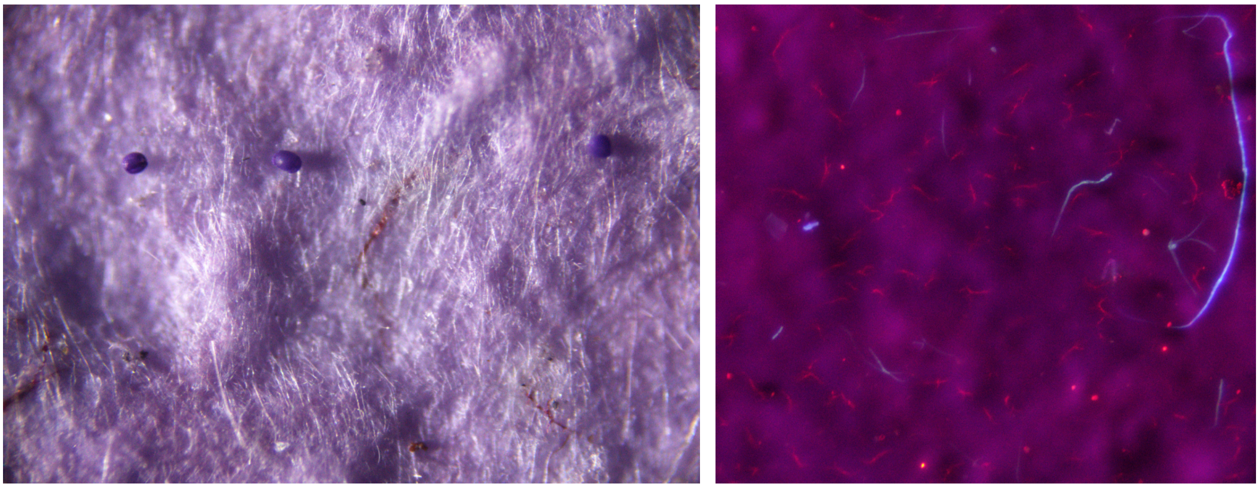


***Fig.4.17.** Nanobeads after Nile Red staining under LED (right – 10x ) and UV light (left – 2.5x)*

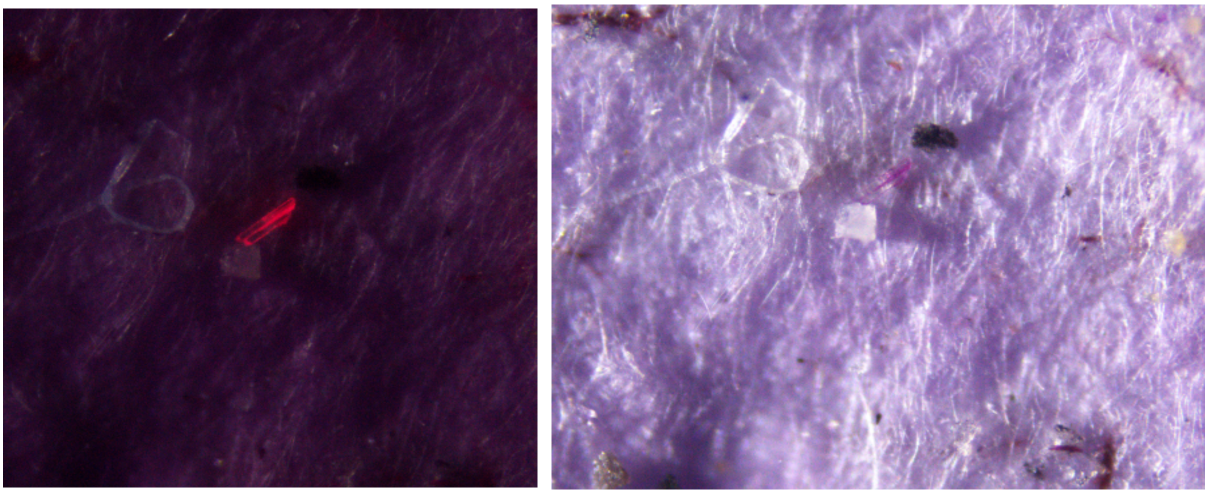


***Fig. 4.18**. Microline after Nile Red staining under UV (left) and LED light (right), 10x magnification*

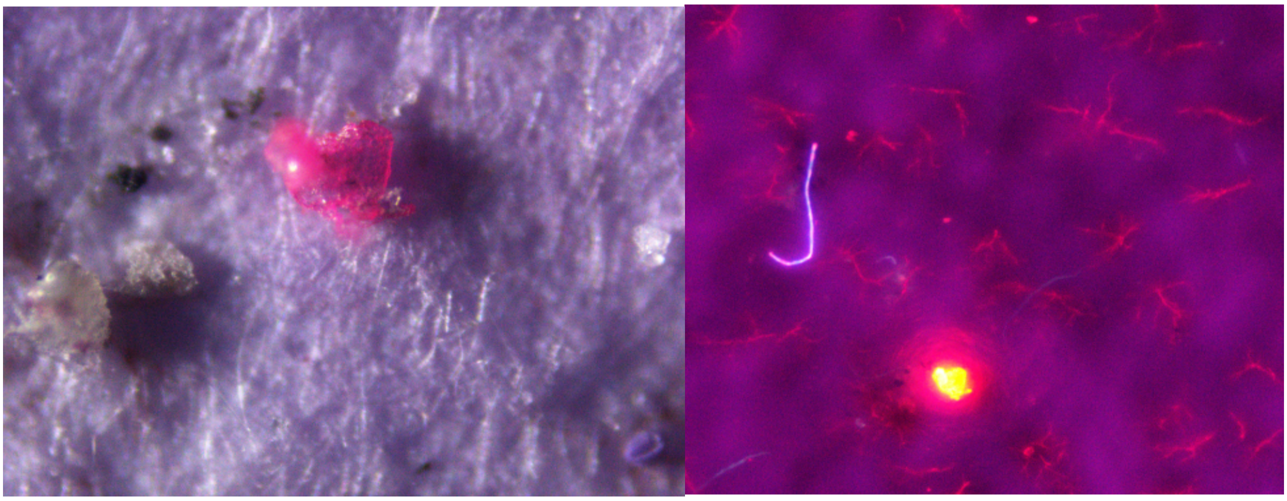


***Fig. 4.19.*** *Microfilm after Nile Red staining under LED (left – 10x) and UV light (right- 4x), and partly colored microfiber (on the right)*

# CONCLUSIONS

In this study, several techniques were described and tested for preparing material for identification and quantification of microplastics in it. Among the different preparation methods are electrostatic separation was used, in addition with the densimetric separation using NaCl solution, to obtain reliable results. Similarly, to reduce the level of error both in the direction of underestimation and overestimation of the amount of microplastics, the organic matter removal step should be used. However, from the analysis executed the organic matter removal on the filter with 15% $H_2O_2$ is not enough to reduce the level of organic material in sand sample. This happen for sample collected from marine environment, while for other environments (for example, cave sediment, glacier) could be sufficient.

An important and necessary step in further research is also the study and development of staining technology using Nile Red or other dyes or their combination for the detection of microplastics (Liu 2021 et al., 2021). In addition various combinations of parameters affecting the staining result (NR concentration and type of solution, staining time and temperature in oven, etc.) need of insights. In particular the use of green-yellow filters/lasers could show the highest efficiency in this type of examination. Moreover, a semiautomatic or automatic counting process, using, for example programs such as ImageJ, could reduce the time analisys, make easier the calculation and reduce the error of the operator. The mandatory verification of particles previously assessed as microplastics and plastic-like particles, both under the UV microscope and after the staining procedure is however necessary before image analysis.

The heterogeneity of methods in literature, the high variability of plastics and subjectivity of the operator for distinguishing microplastics from plastic-like particles (on which identification under microscope is based) make the identification process not only difficult and time-consuming, but also lead to a high probability of error in determination. These kind of errors increases with a decrease in particle size. This happen mainly for the particles with dimensions lower than 0.5mm, which was reported to be the most frequently occurring in this study - 647 of the total 743 particles, identified as microplastics, were found in the category size below 0.5 mm.

While, from the microscope identification of the sediments analyzed a higher concentration of microplastics was found in the zone located about 24 m from the sea - 4.27 MPs/g, compared to 3.1 MPs/g (zone near the shoreline). Thus, the sediment distant from the sea is more polluted than the near one. The most common type of microplastic is a fluorescent fiber that reach the 28% of the total number of microplastic particles found.

For these reasons identification with a UV microscope with UV light, as well as with or without Nile Red, can be considered only a preliminary step in the framework of identification, but not the final one. Nile Red seems to improve the MPs identification than the technique that use only a microscope with UV, but it is still capable of staining some organic materials. Thus, microscopic identification is only a first step or screening (Fischer et al 2016) to discard obvious and easily identifiable objects that are definitely not microplastics and consequently reduce the subsequent amount of work by using more reliable instruments. Different combinations of analytical tools (SEM, FTIR, Raman) can be applied to confirm the MPs particles.

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