MICROPLASTICS-Protocol Proposal DEAKIN UNI – LABTER-CREA – GLOBE ITALIA

Scope

Measure the concentration of microplastics in surface waters.

Overview

Students will measure the concentration of microplastics in surface waters in the chosen hydrosphere study site. The students will apply a simple sequence of operations, using instruments and tools common in high schools (microscopes) as well as in sustainable development education centres. In some cases, self-made tools can also be used as support tools for sampling.

Student outcomes

Students will learn to:
- recognise the main types of polymers and plastic materials in use and in the market;
- categorise plastics dispersed in the environment by size and shape,
- identify sources of microplastics,
- distinguish objects of natural and man-made origin present in surface waters,
- use available technology (filtering units and microscopes),
- calculate the concentration of microplastics in their Study Sites,
- communicate the results of the investigation to the local communities, to other schools in the GLOBE network, to the schools in their own territories,
- collaborate with other GLOBE schools (within or outside their own Countries), and
- share their observations, uploading the data to the archive which will be built by the proposers.

Scientific Concepts

Materials Science

Polymers are substances obtained from the reaction of molecules with one or more reactive functional groups, can be of natural or synthetic origin.

Plastics come from mixing polymers, typically of synthetic origin, with additives that aim at improving their properties.

Microplastics are any pieces of plastic or synthetic polymer material or textile fibres with size between 5 mm and 1 micrometre.

The majority of microplastics dispersed in the environment originates through the fragmentation of large plastic objects during or after use, atmospheric agents or marine/water agents.

Microplastics might also come from personal hygiene or home care products, in which they are contained as already small (micro) plastic particles.

In water, microplastics can be mistaken for microplankton by marine life, thus entering the food chain.

Microplastics can also act as rafts for bacteria and other microbial forms as well as persistent chemical substances, which become “transported” through the movement of water due to currents and winds.

Over time, and under specific conditions, plastics, including microplastics, can release un-reacted monomers and additives that might present a hazard to living things.

Every element, microplastics included, can move between the various containers (biosphere, lithosphere, atmosphere and hydrosphere).

Physical sciences

Typically, the density of plastics is greater than that of water, apart from some plastics that show lower density. The former typically sink in water, but their density can be lowered if they absorb air or other gases and become transportable by the currents over long distances (they don’t sink quickly). The latter typically float, unless they adsorb materials (biofilms, bio-mortar, bacteria, etc.) that cause their density to increase.

Chemicals absorbed on the surface of plastics can change their wettability. Increased wettability causes plastics to distribute more easily across the water column.

The majority of microplastics is hydrophobic. This property too favours their floating, due to interactions with water’s surface tension.

After a period of residence in water, microplastics can become substrates for the deposition of hydrophobic substances, soluble hydrophilic substances, or amphiphilic substances (hydrophilic and hydrophobic at the same time, such as detergents, protein, fatty acids, etc.). The adsorption of hydrophilic or amphiphilic substances on microplastics favours their sinking in water, as their wettability is increased (hydrophilicity). The adsorption of hydrophobic substances on microplastics favours their flotation, through decreased wettability. The adsorption of substances on microplastics often favours biological growth on their

surfaces, further enhancing their wettability and often their density, hence their tendency to sink.

The mechanical action of waves, the oxidising action of air and the action of solar rays degrade plastics, further facilitating their fragmentation.

Earth and Life Sciences

The traditional Earth compartments now have a new companion: one of synthetic origin that is expanding and is modifying the morphology and the quality of the others. The Plastosphere.

The presence in most seas and oceans of islands of rubbish the size of large nations is putting at high risk the equilibria of aquatic ecosystems and threatening aquatic life itself.

Organisms can only survive in those environments that cater for their needs. Earth has many different environments that support different combinations of organisms.

All organisms must be able to obtain and use the resources, all while living in an ever-evolving environment..

Humans can modify natural environments, sometimes with negative effects.

Scientific Investigative Abilities

- To use a filtering device typically employed for microbiological analysis of surface waters.
- To use a digital or simple optical microscope.
- To identify research questions to which an answer can be found.
- To design and perform scientific investigations.
- To use mathematical instruments suitable for data analysis.
- To develop descriptions and forecasts based on evidential proof.
- To recognise and analyse alternative explanations.
- To communicate procedures, descriptions and forecasts.

Level

Intermediate and High Schools.

Time

Preparation of materials: 20 minutes
Sampling: 5 minutes
Filtration: 10 – 30 minutes, function of the turbidity of the sample and of the vacuum system.
Preparation of laboratory blanks: 10-30 min
Analysis of the filters under a microscope: 20-30 minutes.
Recording of results: 5 minutes
Discussion of results as a research team: 1 h

Frequency

Every 6 months.

Materials and tools

Hydrosphere Investigation Data Sheet
Microplastics Protocol
Microplastics Data Sheet
Microplastics Protocol Field Guides
Microplastics Protocol Laboratory Guides

Sampling:
- Telescopic sampling rod or bucket with rope
- Plastic (polyethylene) bottles 500 mL
- Bottle Carrier
- Gloves (vinyl or latex)
- Labels
- Permanent marker, pen, or pencil
- Camera or smartphone to record coordinates and other important features of the site (If the Study Site has already been defined the coordinates are already recorded).
- The camera is used to prepare a Photo-report of the activity but also to record the features of the Study Site in each cardinal direction).
- Digital or alcohol-filled thermometer
- Clip-board.

Filtration:
- Filtration Unit
- Syringe, 50-60 mL
- Latex, rubber or PVC tubing
- OPTIONAL: 1 x three-way connector with 2 x one-way valves to connect the tubes to the syringe and the filtration unit
- OPTIONAL: vacuum line and connectors
- Membranes (47 mm diam., pore size 0.45 μm, cellulose esters, gridded preferred)
- Petri dishes (55 mm diam.)
- Tweezers
- Spray bottle containing deionized water
- Permanent marker
- Labels

Observation:
- Optical microscope (with an at least maximum magnification of 160x), preferably with a connected camera
- OR: smartphone microscope converter
- Calibration grid (glass slide or other)
- Tweezers

Preparation

Suggested Activities: Learning Activities for Microplastics

TEACHER SUPPORT

Microplastics: Protocol – Introduction

The knowledgeable execution of this Protocol requires students to be familiar with:
- the sub-units of measurement of length,
- the concepts related to polymers and plastic materials
- the dimensions and sources of microplastics,
- potential microplastics categorisation schemes,
- typical morphological features of microplastics and plastics.

This introduction is aimed at familiarising teachers (and students) with concepts and information considered necessary to knowledgeably undertake the search for microplastics in surface waters using this or other protocols.

The sub-units of length measurements: sub-units of the metre

The sub-units of the metre are many. For the scope of this work we will limit to: millimeter, micrometre and nanometre. A millimetre is the thousandth part of a metre and is indicated with the "mm" symbol. A micrometre is the thousandth part of a millimetre and is indicated with the "µm" symbol. A nanometre is a thousandth part of the micrometre and is indicated with the "nm" symbol.
It follows that one metre corresponds to 1000 (10^3) millimetres, to 1,000,000 (10^6) micrometres and to 1,000,000,000 (10^9) nanometres.

$1m = 10^3$ mm $= 10^6$ µm $= 10^9$ nm

In other terms:

1m = 1k mm = 1M µm = 1 G nm

To provide you with an idea of the dimensions at play, bacteria and unicellular organisms typically range respectively between 0.1 and 1 µm (bacteria) and 1.5-10 µm (unicellular organisms). In general, viruses are much smaller than bacteria. Most viruses studied to date have characteristic dimensions between 20 and 300 nanometres. Some filoviruses can be as long as 1400 nm, but with a very small diameter (approx. 80 nm). Atoms have dimensions around 10^{-7} mm (0.1 nm, or 1 Angstrom), atomic nuclei around 10^{-11} mm, etc.

Plastics: what are they?

Plastics are typically artificial products, non-existent in nature, synthesised starting from substances that can be found in nature such as oil, coal, some biopolymers etc.

The main structural components of plastics are polymers (from Greek, "made of many parts"), macromolecules obtained through reactions of a molecule with other molecules, either identical to itself or different, a very high number of times. These starting molecules are called "monomers" ("made of one part only").

Some monomers (ethylene, propylene) can be obtained from oil fractions, more or less dense, through processes of steam cracking (breaking of molecular bonds of the starting molecules at very high temperature and in the presence of steam, resulting in the production of molecules of lower mass). The majority of monomers used is constituted of intermediate chemical products (styrene, vinyl chloride, acrylonitrile, etc.), which can be obtained starting from the basic products of the oil industry (ethylene, propylene, benzene, etc.) through reaction with other chemical substances.

Some monomers can react with identical molecules thanks to the presence of double bonds (ethylene, propylene, styrene) or of functional groups of different nature in their structure (amino acids, etc.). Long molecules result, in which the structural units are repeating, sometimes in a random arrangement if there are different monomers that are used. The reaction that involves monomers and results in polymers is called a polymerisation reaction. There are several different types of polymerization reactions.

As the number of monomer molecules participating in the polymerisation reaction increases, so does the mass of the macromolecule (polymer). At the same time the physical state of the polymer might change: it might go from a very low viscosity liquid to one with high viscosity, or to a solid.

Note: typically, not all the monomer mass present in a polymerisation reactor participates in the reaction, with the result that polymers can incorporate a small percent mass of the starting monomers. These can remain in the polymer mass during processing. These remaining monomers can migrate with time to the surface of plastic objects that we use. This fact is particularly significant for food containers used in hot conditions, whose contents can be contaminated by monomers which may migrate into foods. This migration is favoured by time and temperature, as well as by mechanical shaking and contact with certain foods, etc.

Note: polymers are at the core of other materials, which have very wide application and are traditionally not classified as plastics: elastomers or rubbers, and fibres, synthetic, natural or artificial. Rubber based materials are sources of microrubbers, also here considered as part of the microplastics category. **Rubbers, elastomers, synthetic and artificial polymers and other similar compounds and mixtures are considered as plastics in this document, as they pose the same risks as traditionally-defined plastic objects in the environment.**

Rubbers are an important class of materials. Among the synthetic rubbers we highlight those based on butadiene and styrene, butadiene and acrylonitrile, chlorobutadiene, polyesters, silicones. Natural rubber (known as India rubber) is from the Caucciu' plant. Its structural unit is isoprene.

Some polymers are used to make fibres that we may use in clothing or other every day equipment and materials. Fibres used in textile applications can be defined as:

- Man-made, comprising of synthetic fibres (made from synthetic polymers) and artificial fibres (made from regenerated naturally occurring polymers such as cellulose),
- Natural fibres among which: cellulose fibres (cotton, linen, hemp, etc.) and animal fibres, (wool, alpaca, cashmere, etc.).

Among the synthetic fibres we highlight those most common: acrylic (polyacrylonitrile), aramidic (Kevlar), polyester (polyethyleneterephthalate), polyamides (Nylons), polyolefins (polypropylene, etc.).

Among the natural fibres we highlight those with vegetable or cellulosic base (cotton, linen, hemp, jute, etc.) and those of animal origin (wool, silk, bissus, a very rare filament produced by the Pinna Nobilis bivalve mussel of the Mediterranean) and those of artificial origin, obtained from natural renewable substances such as cellulose, through chemical processing (acetate, rayon, viscose, etc.).

Natural polymers of great importance for life are polysaccharides, chains of nucleic acids, proteins and polypeptides.

Note: Polymers, also called resins, very rarely have the properties that applications require. To lift the properties of polymers, they are compounded (mixed) with other substances, called additives, that improve their plasticity, workability, structure and other characteristics such as their stability against ultraviolet radiations, oxidising agents, their ability to be antistatic (dissipating electrical charge) or to conduct electricity. Among the most common additives are: flexibility-enhancing and plasticising agents, dyes and pigments, adhesion enhancers, nucleating agents, flame retardants and lubricants.
Note: just like monomers, even additives can migrate from plastics; some can have very serious effects if they enter the food chain.

Plastics: Brief History, Production and Consumption

Brief History

The early examples of plastics date back to the end of the 1800s. The "plastic century", though, is the 20th. It starts with the discovery of a polymer based on phenol and formaldehyde (1907), patented three years later as Bakelite (in honour of the inventor). Other polymers will shortly follow, based on vinylchloride (1913) and cellulose (1913). They will be heavily used many years later in many plastic products. Nylon was first synthesised in 1935 (poliamide base). It opened the era of synthetic fibres. Nylon later found great uptake in the production of stockings and parachutes used in World War II.

Polyethyleneterephthalate (PET) was patented in 1941. It's the basis of very successful synthetic fibres still currently in use. PET is present in fabrics in the form of polyester fibres (on their own such as in fleece and sportswear, as well as in blends with cotton) and, from the 1970s, in plastic drink bottles. In the 1950s other polymers based on formaldehyde made an appearance, alongside the more common polyolefins such as polyethylene and polypropylene. The latter have literally invaded the world, through their broad spread use in everyday items. In the 1960s design, fashion and art catalysed the high valuation of plastics, only used in items of low cost prior to that time. The following decades saw a flourish of new products, in particular technopolymers, thought of for increasingly sophisticated and important applications.

Lastly, and not to be forgotten, are the most common applications of plastics: clothes and paints.

In less than a century, plastics have invaded our homes, our cities and – we can say – the whole planet, thanks to extraordinary properties. It's these same properties that make plastics one of the most pressing environmental concerns.

Production, consumption and presence in the Environment

WWF estimates that the global plastic production amounted to over 300 million tonnes per year in 2017. This value has grown by an order of magnitude over the last 50 years, when the estimate production for 1964 alone was 15 million tonnes.[1] WWF also estimates that at least 8 million tonnes end up in seas and oceans every year and that oceans may now contain more than

150 million tonnes. Should nothing be done to reduce plastic production, it's estimated by WWF that oceans in 2025 could have a population of 1 tonne of plastic debris to 3 tonnes of fish, and that by 2050 the mass of all plastics would be greater than that of fish. The estimation of the total plastic debris in the oceans and waterways is complicated by the difficulty in measurement and by the inhomogeneity of measurement methods. [2]

Recent reports describe plastic findings in all sorts of environments: glaciers,[3] mountains,[4] even Mount Everest,[5] the Mariana Trench, down to 10 km in depth.[6, 7]

It comes to little surprise that experts have started to identify a new Earth Era: Anthropocene.
This Era is characterised by the pervasive human intervention on ecosystems, and by the relenting pressure exerted by humans on all natural systems.
Experts have also recently characterised plastics as a "techno fossil"[8], found to be present in geological stratifications.

Rocks defined as plastiglomerates[9] have been discovered in Hawaii and are so named as they contain plastic particles inside their mass.

Oceans and seas of the world are invaded by plastic debris. [3, 5, 10-12] The Mediterranean sea for instance has been defined as a Plastic Soup in a Nature Scientific Reports article from 2016.[13] This report estimates that the presence of plastics floating in the Italian seas, especially the portion between Sardinia and Corsica amounts to approximately 10 kg per square km.
These values go as low as 2 kg per square km around the Southern coasts of Italy. These numbers are much higher than those reported for the "Great Pacific Garbage Patch", an area approximately 1 million square km. In this area the concentration of plastic objects is estimated to be 335.000 per square km. In comparison, Mediterranean numbers are up to 1.25 million per square km.[14]

The Classification of Plastics of small Dimensions that are dispersed in the environment

Plastics that are of small dimensions and are found in the environment can be classified in several ways. For the scope of this protocol the main dimensional classification frameworks are discussed here.
There is inhomogeneity in scientific publications in regard to dimensional classification of plastics, in particular microplastics. However, there now is general consensus on setting to **5 mm** (millimetres) the upper dimensional limit for **microplastics**. It is to be noted this approach is not canonical, as the value of 5 mm belongs to the millimetric, not the micrometric scale in the International System of measuring units (SI).
The lower limit for microplastics is still being debated, especially with the emergence of techniques that can detect plastic fragments below the 1 micron level. However, plastic fragments smaller than 1 micron are now typically recognized as nanoplastics. Literature reports different lower limit values, typically dictated by the methodology used for the sampling and preparation. Commonly the limit is set at 330 µm, the typical dimensions of the "Manta" nets that are typically used for the collection of neustons (micro-organisms that live at the water-air interface). These techniques capture particles that float in the first few cm of the water column. Particles of greater density tend to "escape" this measurement, as they tend to sink and end up in the sediments, thus escaping the techniques that are based on neuston and plankton analysis. Other methods use nets with 200 µm openings. In both cases particles of smaller dimensions (micrometric) are excluded from the collection, as they may escape the net through its large openings.

While the scientific community reaches an agreement on the dimensional classification such that it can be reported in the International System of Measurement Units, this protocol adopts the classification of marine debris proposed by the US National Oceanic and Atmospheric Administration (NOAA)[15] and shown in Figure 1. This is a purely conventional classification that categorises marine plastic debris as:
1. Megaplastics, dimensions ≥ 1m
2. Macropalstics, dimensions < 1m and ≥ 2.5 cm
3. Mesoplastics, dimensions < 2.5 cm and ≥ 5 mm
4. Microplastics, dimensions < 5mm and ≥ 1µm
5. Nanoplastics, dimensions < 1µm

As mentioned, it is convention in surface water analysis to adopt 5 mm as the upper dimensional value for microplastics. This protocol follows the same convention, now quite globally adopted.

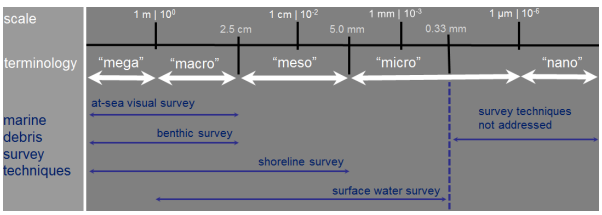

Figure 1. *Dimensional scale for marine debris classification and typical ranges relevant to different techniques. Reproduced from "NOAA, Marine Debris Monitoring and Assessment: Recommendations for Monitoring Debris Trends in the Marine Environment".[15]*

Figure 1 also shows the dimensional intervals of the objects typically researched by the various marine analysis methods:
- At-sea visual Survey
- Benthic Survey

- Shoreline Survey
- Surface Water Survey

Another classification of microplastics is that related their origin. Primary microplastics comprise of particles, pellets, fibres films and/or plastic foams produced at the source in dimensions < 5 mm. Secondary microplastics are formed as the result of fragmentation events of larger plastic debris dispersed in the environment. The generation of secondary microplastics can be due to a variety of causes, including use of plastic objects.

Among primary microplastics are:
- plastic microspheres used for personal hygiene and make-up (glitter and scrubs, toothpaste, detergents, etc.), that are meant to contribute positively to the cleansing act, by contributing as abrasive particles in processes such as scrubbing;
- pellets, small particles such as drop-like, small cylinder or disc, or other shapes, which are made up of a polymeric mixture (often with additives), used as primary ingredient in the manufacturing of plastic objects;
- polymeric textile fibres released through use or wash of fibrous materials (including non-woven materials such as insulating panels and wipes, clothing, and natural fibres used in textiles, etc.).

Distribution of plastic fragments in marine and riverine ecosystems.
Numerous reports have described how plastics can be found at all depths of the water column. Different sampling approaches should be deployed to sample to different depths, and a holistic approach should ideally be taken to understand the distribution of microplastics at the interface between atmosphere, hydrosphere and pedosphere, and their mutual interactions. It is important to note that the distribution of microplastics in littoral waters may also differ from day to day as a consequence of wave action or tides (lifting debris otherwise settled), or varying input from rivers.
We refer to the extensive literature available on the matter.[2, 10-12, 16-23]
This protocol aims at determining the concentration of microplastics present in surface waters. While concentrating on surface waters, we wish not to disregard or underestimate the negative effects on the environment of microplastics denser than water that might be distributed along the water column and the sediment. These negative effects can be grave, such as is the case with coral reefs.[21, 23, 24]

The only aim of this document is to provide a protocol accessible by schools and in line with best scientific practice, that enables students and teachers to generate community-relevant microplastics pollution data.

Problems Caused by Microplastics in the Surface Water Ecosystems
Plastics can reach surface waters in a variety of ways. They can be transported by rivers, surface runoff or wind (especially the finer and higher surface area particles). They can also arrive through more direct human channels: direct input of sewerage, sewerage water treatment plants (many microplastics are too small to be captured in water treatment plants), ballast tanks or rinse water off passing vessels, as well as by direct negligent action by humankind (input of visible solid waste into the environment). [3, 11, 19]
Plastics can be degraded by the mechanical action of waves, winds, sand, as well as by the thermal expansion/contraction effects due to temperature changes, these caused by the electromagnetic effect of solar rays. They can also be degraded by the action of microorganisms. Through these degradative processes plastics in the environment are progressively more severely fragmented to micro and nano dimensions.

The damage caused by macro and meso plastics on the marine ichthyofauna are well known: large fish, turtles and mammals remain trapped in fishing nets abandoned or lost at sea. Turtles, fish and birds eat fragments mistaking them for jellyfish or small preys.[10, 20, 25, 26]

A 2023 report has defined “plasticosis” as a new “plastic-induced fibrotic disease”,[27] that affects the gastrointestinal system of sea birds. The article reports highly concerning evidence that plastics, including microplastics, can cause severe disease. Possibly greater damage, in great part yet to be deeply studied, is that caused by microplastics.

We summarise here an article by the Italian Higher Institute of Health (Istituto Superiore di Sanita’, Italy) (F. Tommasi, *Notiziario dell’Istituto Superiore di Sanità, Vol. 30 n. 6 June 2017)* which examines potential and effective damage exerted by microplastics on the hydrosphere.

“Microplastics are a predominant feature of marine littering. This is due to plastic’s persistence and its hard-to-arrest accumulation. This is the unfortunate consequence of the inability to invert waste management trends and to manage the problem at a large scale.[28-31] All marine habitats, at all trophic levels, are impacted by the presence of microplastics, and by plastics in general: from cetaceans to the smallest possible life forms. Large mammals, reptiles and fish can become trapped in plastic debris, which causes reduced mobility and may result in death by starving or suffocation.[22, 28-31]

The most pervasive problem is constituted by microplastics pollution. Microplastics have characteristic dimensions similar to those of plankton or small crustaceans. They can be mistaken for food by both small and large species. Cosmetic particles such as those used in scrubbers can be mistaken for eggs and smaller plastic debris for plankton. Ingested plastics can cause occlusion of the digestive apparata of the swallower species. This results in starvation, and or infection as the animal loses the inpulse to feed and becomes weaker.

The consequences of increased mortality have repercussions across the food chain, as the overall amount of fish available is reduced, or becomes extinct for some trofic links. These consequences affect the marine as well as other ecosystems.

Microplastics that enter the marine environment as already small (<5 mm), or that become small through fragmentation, become vectors (carriers) of polluting chemicals, especially those residual from manufacturing. Some of these, see phthalates, have severe endocrine consequences. Other types might be pollutants that are present in the marine environment and accumulate on the surface of microplastics due to ideal surface energy conditions. Among these: weed-killers, antiparasitic compounds, metals and metalloids, persistent organic pollutants (known as POPs). Microplastics become rafts for these species and their ability to adsorb chemicals per unit of their mass increases as the particle size decreases and their surface roughness increases.

In practice it has been measured that microplastics carried by polluted waters can concentrate the present pollutants by a million times their measured concentration as species dissolved in the water.[30, 31]

The debate is now as to whether this high level of concentration may lead to the same level of accumulation in intestinal and fat mass, the recognised storage tissue for lipophilic PoPs.[30-32]
The very limited data and study available at this time indicate that the desorption of some common contaminants in the aqueous environments is faster in the presence of gastric juices especially in hot-blood species. This is the case for (polycyclic aromatic hydrocarbons, phthalates, perfluorooctanoic acid (PFOA) and DDT). The desorption appears to be as high as 30 times that measured in marine waters, with consequent accumulation in adipose tissue.[30-32]"

Note: due to low biodegradability and high persistence in the environment, coupled with a low density difference in respect to water, microplastics act as rafts for chemicals but also pathogens. They can transport these for great distances and offer them protection, from UV radiation for instance, thus granting pathogens greater chances of survival. Relevant literature is in good agreement on the noxious fall-out effects due to the ubiquitous presence of microplastics.[10, 12, 20, 21, 23, 24, 26, 30, 33-35]

"Human consumption of marine and riverine products puts humans at the last step of direct exposure to this type of pollution. The scientific literature has analysed at length the impacts on the impoverishment of habitats and marine life in general. The known effects of microplastics ingestion directly or indirectly through seafood are quite unknown and positions are very controversial, given the complexity of interplaying factors and the difficulty to backtrack and identify the single pollutants. This causes great uncertainty in determining the levels of human exposure to aqueous pollutants, vectored by microplastics.[22, 30-32]"

The Plastisphere

In 2013 Erik Zettler, Tracy Mincer and Linda Amaral-Zettler published a seminal article on the interactions between plastic debris and microbial communities, coining the term "Plastisphere".[34, 36] The Plastisphere was defined in the title of the article by Zettler et al. as "microbial communities on plastic marine debris". The plastisphere comprises of the complex mix of microbial inhabitants (ecosystem) which progressively colonise plastic debris in the environment, not just marine water. The abstract highlights that plastic debris can cause biological imbalance: "*Plastisphere communities are distinct from surrounding surface water, implying that plastic serves as a novel ecological habitat in the open ocean.*".[36] This observation is in line with previous observations that floating plastic debris can transport harmful algal species across ecosystems.

Zettler et al. demonstrated that these microbial communities interacted with the plastic debris in chemical and mechanical ways, highlighting signs of erosion due to exposure to chemicals that are able of attacking hydrocarbons. Further, they described how opportunistic pathogens were found in some samples. More recent publications describe the matter more in detail, indicating that DNA mapping techniques are accelerating knowledge building in terms of the plastisphere.[34] The nature of the plastisphere is only beginning to be understood for the many different types of marine debris, but the effects of the plastisphere as a dynamic actor on the surrounding environment are largely not understood.

The UN's Turning Off the Tap Report

The United Nations Environment Programme has issued a report that recognises plastic flows as posing a threat to life on Earth, in mechanical and chemical terms.[37] The complexity of managing the flows of plastics is extremely high, and requires efforts that range from education to industrial transformation and are underpinned by monitoring activities.
This report will form the basis for global coordinated change, but needs to be supported by widespread (geographically and temporally) measurements to monitor the inputs of plastics in the environment, as earlier highlighted by GESAMP as critical to measuring progress.[38]

Microplastics Monitoring Protocol - Background

Sampling and analysis methods used for the determination of microplastics. Strengths and weaknesses.

Microplastics sampling, preparation for analysis and analytical/quantification methods – brief introduction

Several methods have tackled the measurement of microplastics in water bodies. All protocols consist of three phases: sample collection, preparation of sample for analysis, and analysis.

Sample collection can be of two types: mass sampling and reduced volume sampling.

Sample collection

Reduced volume sampling

These methods (the majority) rely on the continuous filtration of large volumes of surface water samples.
A net is typically dragged just below the water surface for a set distance, and the water being sampled is continuously filtered through the net. This method results in the accumulation of debris in the net and in a chamber at the downstream-most point of the net.[38]
These methods are typically well suited for marine waters, lakes and lagunes. Reduced volume methods allow to sample large bodies of water, hence producing quite robust data that describe the situation over a large sampling surface. This is very useful for the collection and analysis of macro and mesoplastics but is known to suffer from the loss of the smaller fragments due to the size of the mesh. Mesh openings are typically in the few hundred-micron range. This is necessary to ensure sufficient water flow through the net and loss of microplankton (which reduces downstream processing). The major exponent of this class of methods is the NOAA Protocol (https://marinedebris.noaa.gov/sites/default/files/publications-files/noaa_microplastics_methods_manual.pdf).[15]

The NOAA protocol uses a "Manta", a rectangular section net, dragged behind a vessel at a speed of 2-3 knots. The openings in the mesh that constitutes the net typically measure 330 µm. A flowmeter allows to measure the volume of water that has been filtered. At the end of the sampling event, the net is collected and inverted to collect the sample. Rinsing of the net is followed by the rinsing of the collector placed at the end of the net.

The major benefits of this approach are its robustness and its ability to sample large water surfaces. The main drawbacks are of sampling and logistical nature. These methods typically only collect plastic fragments larger than approximately 100-300 µm (depending on the mesh size of the chosen net). This concern has been confirmed as well-warranted, as recent literature has confirmed that large mesh size (or nominal pore size if using filters) in sampling or sample filtering might cause an under-representation of the number occurrence of microplastic fragments in all water bodies.[39] The main logistical challenge presented by the NOAA and similar methods, though, relates to the need for specialised vessels (and, at times, chemical processing and high-definition chemical analysis – described later) which limits their deployability in schools and citizen-science scenarii.
A secondary logistical challenge is the difficulty to deploy these methods close to shore, at the interface between biosphere, hydrosphere, pedosphere and atmosphere.

Alternative reduced-volume methods that are emerging include the use of pumps and hydrocyclones, to take advantage of density differences to separate water particles. These are more broadly deployable but require the use of bespoke equipment. Nevertheless, the accuracy and precision of these methods in determining microplastics concentrations appear high.[40]

Lastly, other emerging sampling methods offer options for contemporaneous sample collection and analysis in continuous flow, but most are under development and very little literature is available (hence not reported here).

Mass sampling

These methods involve the collection of a pre-determined volume, and do not require on-site filtration. Volumes of up to 50 L are typically collected.
These methods are more suitable than reduced-volume methods for close-to-shore surface water sampling and deep-water sampling. Lower-level equipment is required to deploy these methods (typically a sampling device and bottles). These methods are a lot more accessible by a broader class of participants, as they can more easily be adapted to suit schools and the public, thus facilitating citizen science campaigns and broader-scope investigations.

Sample preparation before analysis

After collection, the sample is prepared for analysis. The preparation of the sample is typically a function of the sampling method (volume of the sample and the type of solids present in the sample) and of the level of analysis that is to be conducted. The analysis can be physical-property-based (requiring density separation, or physical separation by size, followed by morphological assessment) and/or chemistry-based (requiring density separation, chemical preparation

and further sample preparation and staging for analysis).[38, 41]

Typically, once a sample has been collected, the following steps take place in optional fashion (not all protocols use these):
- separation by size, including the preliminary removal by hand of large particles and natural solids (weeds, etc.);
- density separation;
- chemical attack to remove biological residue.
The last step in particular is deployed when biological solids may interfere with chemical identification of the solids in the sample.

As mentioned, in methods such as the NOAA protocol (described below) the sample is gathered inside a net through active filtration during sampling. As the opening of the manta net is in the tens of centimetres, this method tends to collect large as well as small particulate. The sample is then recovered by inverting and "rinsing" the net, yielding the residue to be analysed. The solids collected are then separated by size using a range of sieves and filters, and by density using high salinity solutions.

Following the NOAA protocol, which we refer to as "best practice", the sample separated in the steps just described is digested using a hot solution of hydrogen peroxide and salts (sometimes with the addition of sulphuric acid). This step degrades the biological component of the sample which could otherwise interfere with the analysis steps to follow. The liquid is then filtered on fine filter membranes and is set aside for morphological and chemical identification.

In mass sampling methods the sample is collected in vessels (preferably glass or metal) with a known volume. The sample is then separated by density or dimensions (filtration) in a specialised laboratory.
Also in these methods, chemical digestion of the biological matter is sometimes applied. The need for such a radical treatment should always be weighed against the characteristics of the sample and the requirements of the analysis. This step can be considered superfluous in the case of "dilute" samples, *i.e.* samples in which the biological debris may not be of concern to the analysis because of its low occurrence or because the analysis to be performed is not of chemical nature.
One must also consider that aggressive chemical treatment may cause the loss of other solids of anthropogenic nature, which could be of interest. Among these are natural textile fibres such as wool, cotton, linen, etc. These are known vectors and indicators of microplastics which are typically deposited on their surface through industrial treatments during product manufacturing (water-proofing, stiffening agents, etc.).

Microfibres of textile origin, of all types (natural, and man-made (artificial, synthetic)) are increasingly becoming of interest to the scientific community because of their abundance, as recently reported in a 2022 NOAA Report on Microplastics Pollution submitted to the US Congress.[42]

Analysis
There are simple and complex methods of sample analysis for the presence of microplastics. Classical simple methods are the morphological or optical-appearance-based methods. These include observing the sample for physical shapes or features (key-based recognition, comparison with library of samples), or for colour, fluorescence, etc. Some of these methods can take advantage of sample staining techniques that provide greater contrast for microplastic objects (see Nyle Red method). More advanced methods include the use of chemical identification techniques such as Raman microscopy or Fourier-transform infrared microscopy.[43]

The simpler methods (optical/morphological identification) offer an excellent starting point for the identification of materials of anthropogenic nature. With low technical requirements (filtration units, optical microscope or microscope attachment for a cell phone), these methods are widely accessible by the public and all school levels.

The more advanced methods mentioned above typically use infrared or Raman spectroscopy. They have high technological requirements, come at a high cost (including the requirement for sample preparation on special substrate membranes), and are not widely accessible. These methods are more suitable for Universities, research centres and environmental protection authorities and for projects that target a reasonably low number of samples, as preparation and analysis can be time expensive but also time consuming.

The high number of sample handling steps is a recognised drawback of most methods described above. Some methods are emerging that minimise sample handling, by coupling sample collection and in-flow analysis. Among these a method that uses ultrasound to identify microplastics in suspension using a principle of sound-density difference.[44, 45] While this method is applaudable in terms of ease of operation, the need for bespoke equipment is the main barrier to its broad deployment outside academic and EPA environments.

We refer to an extensive 2019 report by GESAMP for those seeking greater information on the above methods.[38]

The NOAA and similar methods more in detail

The NOAA method

The protocol described here markedly simplifies the one originally proposed by NOAA for surface waters[15].

This is described in the following document: *Marine Debris Monitoring and Assessment: Recommendations for Monitoring Debris Trends in the Marine Environment, NOAA Marine Debris Program National Oceanic and Atmospheric Administration, U.S. Department of Commerce Technical Memorandum NOS-OR&R-46 November 2013*.

The document above describes a number of techniques, starting from those deployed by Carpenter and others in 1972, who were the first to identify the occurrence of plastics and microplastics in the Sargasso sea.[46] A number of informal attempts are reported, along with mention of the still very few scientific expeditions. The great variability of techniques used is discussed. This variability causes great complexity in comparing the data obtained with different methods. After in-depth review of the available scientific literature, NOAA presents guidelines for the monitoring protocols that are heavily (declaredly) influenced by the *California Cooperative Ocean and Fisheries Investigations (CalCOFI)*.

The guidelines for monitoring as described in the document were first tested in a pilot exercise, to then be deployed in the field with increasing levels of rigour. The later tests examined design criteria, execution and refined equipment needs. The methods of interest to this protocol are referred to in paragraph 3.5.

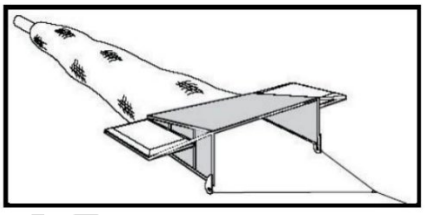

Figure 2. Manta net, reproduced from swfsc.noaa.gov.

The methods specify deployment conditions and materials specifications. The net must be accurately positioned to avoid the wake generated by the vessel. The path that needs to be sampled is determined as 0.5 nautical miles (1 nautical mile = 1.852 km) sailed at a speed of 1-3 knots (0.514 – 1.55 m/s), for approximately 15 minutes. The time of sampling is calculated as the time that the flowmeter has been measuring water being filtered. Figure 2 shows the typical manta net and Figure 3 shows a typical deployment.

Monitoring the aspect of the net during sampling is critical. Net positioning must be monitored, as must be the potential for entry of large items into the net. Further care needs be taken to monitor the track followed (simplified by the use of portable GPS units). To ensure significant data can be produced, three sampling events need to be performed for each water tract. The final result is calculated as the average of the three measurements.

In theory, the volume of filtered water can be derived from manta design data and flowmeter data. In practice, many factors could be contributing to misrepresentation of the sample volume. Among these: wave patterns, incorrect positioning of the net, partial occlusion of the mesh.

While the process can be performed with great accuracy using properly equipped vessels, the difficulties in managing the nets are not to be underestimated. Similarly, the sample collection step is very critical and requires high levels of care.

At the end of the sampling event, the net is recovered inside the vessel and is rinsed using sea water in order to collect all solids into the cup placed at the end of the net. Further difficulty could arise from the potential interaction of debris with the mesh (becoming lost to the measurement and requiring intervention to become dislodged).

Once the sample is collected in the cup, it is then filtered using a series of metal mesh filters (5 mm mesh and 0.33 mm mesh). This step results in two fractions: one of debris of size greater than 5 mm, the second with dimensions between 5 mm and 0.33 mm. This step can be performed on the vessel or in a laboratory and must be followed by rinsing of the sample with deionised water. Should it not be possible to immediately move to the analysis of the sample for microplastics, the sample should be retained in glass containers.

The steps that follow are very delicate and must be performed in a well-equipped and safe laboratory.

1. Determination of the mass of sampled microplastics
2. Oxidation of the organic matter by hot hydrogen peroxide (the most hazardous step)
3. The separation of particles based on density
4. Microscope examination of the obtained material.

Many secondary schools have well-equipped and safe laboratories which could perform the above steps in ideal conditions. Nevertheless, these steps impose significant time commitment that might only be suitable for specific school curricula.

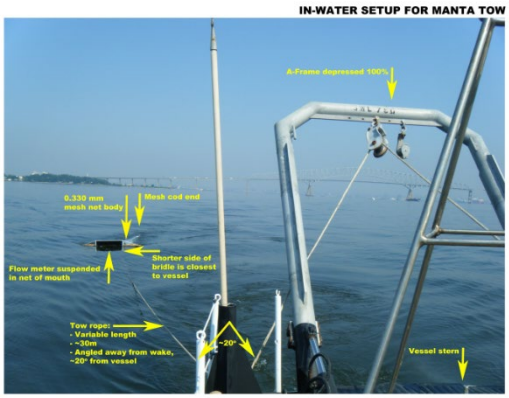

Figure 6. In-water setup for a manta tow. The vessel shown has an A-frame at the stern that is fully depressed, which supports a tow rope that is cleated to achieve and angle of ~20° between the vessel and the net to minimize interaction with the vessel's wake. The shorter side of the bridle should be closer to the vessel to help facilitate avoidance of sampling the wake.

Figure 3. *In-water setup for a manta tow. Reproduced from NOAA: "Marine Debris Monitoring and Assessment: Recommendations for Monitoring Debris Trends in the Marine Environment" NOAA Marine Debris Program National Oceanic and Atmospheric Administration, U.S. Department of Commerce Technical Memorandum NOS-OR&R-46 November 2013.[15]*

The major concerns with the NOAA method (and similar methods) relate to its availability to the public and schools and its ability to be deployed in smaller bodies of water (rivers, creeks, little lakes, ponds). These methods have high equipment and vessel requirements. Such equipment is typically only available to professional environmental protection or monitoring agencies or to Universities and advanced research centres. Schools might be able to gain access to these methods through bespoke partnerships and collaboration arrangements with local agencies and institutes or Universities.

While the "manta net" methods are deployable without change in large lakes and rivers, they can be applied in smaller rivers through the use of bridges (from which the net is lowered and manoeuvred).
In the case of smaller-footprint or more static surface waters such as canals, rivers, creeks and small lakes and ponds the "manta net" methods are impractical.
Lastly, these methods require a reasonably high level of sample handling in the various steps and time-consuming sample preparation. The chances of contamination by non-highly-trained personnel is non-negligible and needs to be taken into account when considering which method can be deployed across a large network of schools, while providing robust, research-quality, data.

<u>Other trawler-based initiatives</u>
The method used by NOAA has been applied by a number of jurisdictions, including EPAs, Universities, Research Centres and major jurisdiction such as the Italian Ministry for the Environment.[47] Adaptations of the method for smaller water bodies have also been implemented across the world with varying degrees of success.[48-50]

We report an extract from an Italian Ministerial document:[51] Modulo 2 del MATTM, il Ministero dell'Ambiente e della Tutela del Territorio e del Mare "*Article 11 of the D.lgs. 190/2010, related to monitoring programs for marine strategy Art. 11, of the MATTM presents the Methodological Guides for the implementation of Conventions agreed to by key Italian agencies (Ministero dell'Ambiente e della Tutela del Territorio, e del Mare e Agenzie Regionali per la protezione dell'Ambiente (ARPA)) in December 2014. In September 2016 the guides were updated. Modulo 2 relates to the Analysis of Microplastics. It lists the parameters to determine in each study site, the relevant instruments and the reference methodology, as reported in Table 1.*"

Sampling is performed first at the water-air interface (surface), then in the basal zone of the thermocline. Consequently, the parameters listed in Table 1 need to be determined throughout the water column.

Table 1. List of parameters to measure at each study site, relevant instrumentation and reference methodology. Reproduced in English from the original version.[38]

	Parameter	Instrument	Reference methodology
Chemico-physical variables	Depth	Multi-parameter probe with fluorimeter	Method as per DM 260/2010, analytical method of reference: ICRAM-MATTM for the monitoring of coastal environment (2001-2003)
	Temperature		
	Salinity		
	Dissolved Oxygen		
	pH		
	Transparency	Secchi disc	
Microplastic analysis	Quantity (microparticles/m^3 of sampled water) per type and colour	Stereomicroscope	Sheet 2

Note: the thermocline is the zone that separates the surface zone (which typically undergoes mixing) from the deep zone in oceans, lakes and seas. In the thermocline you can measure a sharp temperature change between the two zones.

Sampling takes place using a "manta-type" net, built with the purpose of sampling the superficial layer of the water column. This net samples the fraction of water which undergoes frequent remixing through the action of waves, wind and irradiation.
The use of a net allows to sample large volumes of water, retaining materials of interest. The NOAA manta net (Figure 2) is made up of a rectangular metal mouth, to which the net is attached. The end of the cone is affixed to a collector cup. Two empty metal fins, located either side of the mouth, keep the manta net afloat.
The dimensions of the mouth are not fixed. They are instead determined as a function of the size of the trawling vessel. Guidelines regulate the inner dimensions of the mouth: a ratio of 0.5 between height and width. The reference (NOAA) mouth (inner

measurements) measures 25 cm in height and 50 cm in width. The net measures 2.5 m. The overall resulting shape resembles a truncated pyramid. The net mesh size is typically 330 µm. Continuous monitoring of filtration efficacy is necessary to avoid potential problems arising from mesh clogging. Monitoring is particularly important in case of eutrophic waters. The wings provide flotation to the mouth. The dimensions of the wings are a function of the weight of the mouth. Recommended wing length is 40–70 cm. A flowmeter is mounted in the net to ensure the results can yield a volume-based concentration of microplastics. The flowmeter measures the volume of water that is passed (filtered) through the net.

The DEAKINUNI-LTCREA-GLOBE ITALIA method

Sampling strategies for microplastic analysis can be divided in mass sampling and reduced-volume sampling. In reduced volume sampling methods, the sample is collected while its volume is reduced as part of the collection process, retaining only the parts of the sample useful for later investigations. Of these methods, the NOAA method and other net-based methods.
Mass sampling involves collection of a set volume of water without the contemporaneous reduction. The extraction of solids of interest takes place in a second step, typically in a laboratory.

The DEAKINUNI-LTCREA-GLOBE Italia method proposed here falls into this latter category, with the advantage that the extraction of microplastics can be performed directly in the field, immediately following mass sampling. It takes advantage of several similar methods and tries to combine them in such a way to control sample contamination, while maximising participation.

Key features
This method involves three simple steps: 1) sampling of a small volume of water (following GLOBE protocols), 2) filtration, and 3) observation.

This method requires the sampling of small volumes of water (500 mL) in the sampled body of water. The filtration (on the field or in the laboratory) is followed by observation of the filters using an optical microscope and, if possible, more advanced techniques such as Fourier-Transform InfraRed microscopy, if available through collaborations with well-equipped laboratories.

In the method proposed here, samples are collected in the field, using clean equipment and following GLOBE sampling field guides. Special attention is to be paid to reducing the potential for sample contamination.

Three water samples are collected at each sampling point, with a volume of 500 mL each.

The samples do not undergo density separations or chemical treatments. They are instead filtered using a vacuum-driven biology filtration unit, commonly employed in microbiological analysis.

After filtration either in the field or in the laboratory, the samples are stored in clean Petri dishes and observed using either an optical microscope (preferred) or a smartphone, augmented with a microscope-converter lens.

With the help of a *Microplastics Recognition Guide*, the objects identified by the students as microplastics or "unknown" are recorded in the Microplastics Data Sheet.

Where possible, the students record images of the objects they observe. These will help data validation by a teacher or by an expert collaborator, but also help as future training material and data record for each Study Site.

This method minimises sample handling and combines simple, well-established and well-tested techniques that are familiar to many schools around the world.

The surface waters sampling techniques follow GLOBE protocols. The sampling and sample processing techniques used in this proposed method have been in use since the 1970s in European schools, especially in Italy. In specific, this method takes advantage of field-deployable filtration units destined for microbiological analysis. These techniques were introduced by the GREEN (Global Rivers Environmental Education Network) Project started by Michigan University in the 1980s. Lastly, the method uses simple optical microscopes (with low magnification requirements), which are available in most schools. Advanced sample characterisation is not necessary but can be arranged through collaboration with Universities and research centres.

Aside from advanced characterisation options, the method can be performed in whole by Secondary schools, or by Intermediate Schools with the help of Secondary schools.

Materials Recommended for Sampling

The following materials facilitate safe sampling and record keeping. Some of these are optional, such as the telescopic sampling rod.
The recommended materials are:
- OPTIONAL: telescopic rod with sample bottle holder (this helps sample from the shore, but a few metres away from the shore itself);
- Alternatively: bucket with rope for sampling as per GLOBE Sampling accessible at this link: https://www.globe.gov/documents/11865/920675f5-56c0-46a3-97b5-74f9953b2ae4);
- Vinyl or latex gloves;
- 500 mL low density polyethylene bottle with large neck and lid (safer than glass containers for deployment in the field);
- Sample bottle carrier;
- Datasheets;
- Clipboard;
- Digital or alcohol thermometer;
- Labels;
- Permanent marker, pen and/or pencils;
- Camera or smartphone (to document the Study Site)
- Laboratory absorbent paper or chamois to dry the bottles before labelling them.

Materials for Filtration Steps
- Microplastics in Surface Waters Data Sheet
- Filtering unit for biology
- Spray bottle containing deionized water
- 4 rubber stoppers for filtering unit
- Tweezers to handle the membranes
- 1 syringe filter for filtering unit
- Samples
- Individually-packaged (individual packaging is OPTIONAL) membranes diameter = 47 mm (pore size 0.45 µm) gridded, minimum two membranes per sample
- Rigid clipboard
- Pencil, pen or permanent marker
- Syringe 50 or 60 mL (if filtering in the field)
- Labels
- Latex, silicone or PVC tubing
- Camera or smartphone
- OPTIONAL: 1 x three-way connector with 2 x one-way valves (check valves) to connect the tubes to the syringe and the filtration unit
- OPTIONAL: Vacuum line
- Latex or vinyl gloves
- Petri dishes diameter = 55 mm, one per membrane
- 1 L distilled or deionized water

Material for optical identification of microplastics (visual technique)
- Optical microscope (able to reach a minimum magnification of 160x), preferably with a connected camera and laptop (or smartphone)
- OR: smartphone microscope converter with smartphone
- OPTIONAL: Calibration grid (glass slide or other)
- Tweezers
- Microplastics Recognition Guide

Materials for microplastics characterisation (analytical approach – NOT for use in this protocol)
- High resolution optical microscope or fluorescence optical microscope;
- FTIR microscope (Fourier Transform Infra-Red spectroscopy)
- Raman Microscope
- Electron Microscope (destructive method)

GENERAL AND OPERATION-SPECIFIC ADVICE

In chemical and bacteriological analysis of samples great emphasis is given to the possibility of sample contamination. Precise instructions are typically provided to students and teachers to minimise the probability and extent of sample contamination.

The same care needs to be applied when using this protocol. Among the plastics that can contaminate the sample we highlight microfibers, as they are most common in clothing worn by operators and they become often easily airborne.

Contamination can be limited by:
- the operators performing rinsing of sample bottles and sampling equipment using water from close to the Study Site (sampling and disposing of the same downstream from the same sampling location);
- the operators remaining upwind of the sampling tools where possible;
- the storage of equipment (in particular bottles) in closed laboratory cupboards after rinsing and drying when not in use – especially sample bottles;
- measurement of water temperature using one of the rinsing cycles of the bottle while preparing for sampling;
- the use of filtered deionised water for all steps;
- using an air filter on the filtration unit lid;
- closing the bottle, filtration units and Petri dishes as soon as possible after handling;
- storing the dry membrane containing the samples in closed Petri dishes.

Prior to the sampling day, the equipment should be inspected, rinsed in filtered deionised water and let dry in air in a closed cupboard.

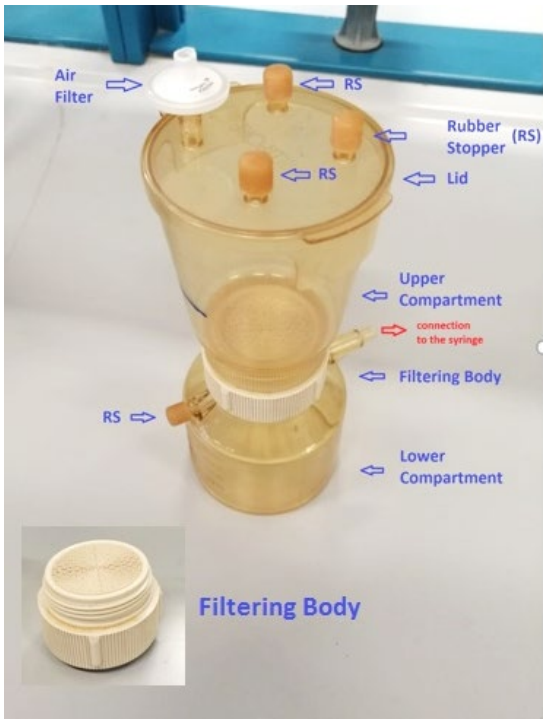

Figure 4. The fully assembled filtration unit with its various components indicated.

The filtering apparatus could vary in design, but typically consists of 3 parts, as in Figure 4:

1. The lower compartment: 250 mL in volume, this is used to make vacuum. It has two connectors used to apply vacuum and it collects the filtered water (to be discarded).

2. The filtering body: this supports the filter membrane. It's added to the lower compartment by applying slight pressure or twisting, depending on the design of the unit.

3. The upper compartment: this is where up to 250 mL of sample will be added. Its lid has 4 connectors. One of these must host a filter for air, the other three can be closed using rubber stoppers. The air filter allows air to enter the unit displacing the removed volume of water, maintaining atmospheric pressure. The role of the filter is to stop air-borne particles from entering the unit and contaminating the sample during the filtration steps. The lid must be only kept open no longer than what is strictly necessary to add the sample. (Figure 4).

During filtration the operator must consider the risk of contaminating the sample during membrane handling and filtration unit opening or handling.

Observation of the samples using a microscope, the last step run by schools, requires care to avoid contamination while extracting and handling the sample membrane from the Petri dish and its positioning on the glass slide on the microscope stage. "Dry runs" with new membranes can be performed with the students to familiarise them with the degree of care required to minimise sample contamination. Equally, "blanks" or "controls" should be prepared and characterised. Examples of blanks:

- A new membrane left on the microscope stage for set periods of time (1-3 h) to observe airborne contamination;
- A filtered deionised water sample
- A sample prepared using deionised water but without applying the air filter on the lid for the filtration unit
- A new membrane let sit in a spot in the classroom over the weekend.

Observing and measuring the extent of contamination that is possible due to airborne (and not only) microplastics is a key aspect of this protocol.

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Microplastics in Surface Waters – Sampling with Bottle only

Field Guide

Task

To collect the samples safely while standing on the banks of the water body (river, creek, lake), avoiding sample contamination. You may also wish to follow GLOBE's Sampling Procedures document which you can find at this link: https://www.globe.gov/documents/11865/920675f5-56c0-46a3-97b5-74f9953b2ae4

Necessary Materials

❑ *Site Definition Sheet (https://www.globe.gov/documents/352961/a3624505-f6c3-4f22-be4c-75d2b6e98d78) (already filled in for the GLOBE Hydrology Protocol)*

❑ *Microplastics – Surface Waters Data Sheet*

❑ Polyethylene bottles, 500 mL with lid

❑ Sample bottle carrier

❑ Rigid clipboard

❑ Permanent pen, pencil or pen

❑ Labels

❑ Latex or vinyl gloves

❑ Chamois, cotton cloth or kitchen paper towel

❑ Camera or smartphone

❑ Digital thermometer

In the field

1. Fill in the *Microplastics – Surface Waters Data Sheet*
2. Wear gloves.
3. Open the polyethylene bottle. An operator will retain the lid with care that it does not become contaminated.
4. Wet the bottle and your gloved hand in the body of water to sample. Also rinse the lid in the same body of water. Discard the contents of the bottle but not directly where you will be sampling.
5. Repeat another two times.
6. Re-immerse the bottle in the body of water, maintaining it at approximately 10 cm below the water surface.
7. Extract the bottle from the body of water.
8. Rinse the thermometer with deionised water.
9. Measure the temperature of the water and immediately close the bottle with the lid.
10. Register the temperature value observed in the *Microplastics – Surface Waters Data Sheet*.
11. Dry the bottle and apply a label containing the sample code, time and date of the sampling.
12. Place the sample in the sample carrier and repeat the procedure twice more.

Microplastics in Surface Waters – Sampling with Bottle and Bucket

Field Guide

Task

To collect the samples safely while standing on the banks of the water body (river, creek, lake), avoiding sample contamination. You may also wish to follow GLOBE's Sampling Procedures document which you can find at this link: https://www.globe.gov/documents/11865/920675f5-56c0-46a3-97b5-74f9953b2ae4

Necessary Materials

❑ *Site Definition Sheet (https://www.globe.gov/documents/352961/a3624505-f6c3-4f22-be4c-75d2b6e98d78) (already filled in for the GLOBE Hydrology Protocol)*

❑ *Microplastics – Surface Waters Data Sheet*

❑ Bucket with rope

❑ Polyethylene bottles, 500 mL with lid

❑ Sample bottle carrier

❑ Rigid clipboard

❑ Permanent pen, pencil or pen

❑ Labels

❑ Latex or vinyl gloves

❑ Chamois, cotton cloth or kitchen paper towel

❑ Camera or smartphone

❑ Digital thermometer

In the field

1. Fill in the *Microplastics – Surface Waters Data Sheet*
2. Wear gloves.
3. Rinse the bucket with the rope in the Study Site water three times, but not directly where you will be sampling according to the Globe "*Collecting a Water Sample in a Bucket Field Guide*".
4. Collect sampling water in the bucket according to the Globe "*Collecting a Water Sample in a Bucket Field Guide*".
5. Rinse the thermometer with deionised water.
6. Measure the temperature of the water in the bucket.
7. Register the temperature value observed in the *Microplastics – Surface Waters Data Sheet.*
8. Open the polyethylene bottle. An operator will retain the lid with care that it does not become contaminated.
9. Rinse the bottle with the bucket water three times. Also rinse the lid. Every time discard the contents of the bottle but not directly where you will be sampling.
10. Fill the bottle to the desired volume with the bucket water and immediately close the lid.
11. Dry the bottle and apply a label containing the sample code, time and date of the sampling.
12. Place the sample in the sample carrier and repeat the procedure twice more from point 3.

Microplastics in Surface Waters – Sampling with Bottle and Sampling Rod

Field Guide

Task

To collect the samples safely while standing on the banks of the water body (river, creek, lake), avoiding sample contamination. You may also wish to follow GLOBE's Sampling Procedures document which you can find at this link: https://www.globe.gov/documents/11865/920675f5-56c0-46a3-97b5-74f9953b2ae4

Necessary Materials

❑ *Site Definition Sheet (https://www.globe.gov/documents/352961/a3624505-f6c3-4f22-be4c-75d2b6e98d78) (already filled in for the GLOBE Hydrology Protocol)*

❑ *Microplastics – Surface Waters Data Sheet*

❑ Telescopic rod for sampling

❑ Polyethylene bottles, 500 mL with lid

❑ Sample bottle carrier

❑ Rigid clipboard

❑ Permanent pen, pencil or pen

❑ Labels

❑ Latex or vinyl gloves

❑ Chamois, cotton cloth or kitchen paper towel

❑ Camera or smartphone

❑ Digital thermometer

In the field

1. Fill in the *Microplastics – Surface Waters Data Sheet*
2. Wear gloves.
3. Open the polyethylene bottle. An operator will retain the lid with care that it does not become contaminated.
4. Extend the sampling rod to the maximum length allowed.
5. Put the bottle in the sample holder at the end of the sampling rod.
6. Wet the bottle in the body of water to sample. Also rinse the lid. Discard the content of the bottle but not directly where you will be sampling.
7. Repeat twice more.
8. Re-immerse the bottle in the body of water, maintaining it at approximately 10 cm below the water surface if possible.
9. Extract the bottle from the body of water.
10. Recover the bottle from the sample holder
11. Rinse the thermometer with deionised water.
12. Measure the temperature of the water and immediately close the bottle with the lid.
13. Register the temperature value observed in the *Microplastics – Surface Waters Data Sheet.*

14. Dry the bottle and apply a label containing the sample code, time and date of the sampling
15. Place the sample in the sample carrier and repeat the procedure twice more.

Microplastics in Surface Waters – Sample Preparation through Filtration using a Syringe

Field or Lab Guide

Task

Prepare samples for the analysis, avoiding contamination. Filtering immediately after sampling avoids the formation of possibly compact deposits at the bottom of the container. These, hard to remove, might cause the loss of useful sample and could negatively affect the measurement.

Necessary Materials

- ❑ *Microplastics – Surface Waters Data Sheet*
- ❑ Filtering unit for biology
- ❑ 4 rubber stoppers for filtering unit
- ❑ 1 syringe filter for filtering unit*)*
  (provided in filtering unit kit
- ❑ Sterile membranes diameter = 47 mm (pore size 0.45 µm), gridded, minimum two membranes per sample
- ❑ Syringe 50 or 60 mL
- ❑ Latex, silicone or PVC tubing
- ❑ Latex or vinyl gloves
- ❑ Petri dishes diameter = 55 mm
- ❑ 1 L distilled or deionized water
- ❑ Spray bottle containing deionized water
- ❑ Tweezers to handle the membranes
- ❑ Samples
- ❑ Rigid clipboard
- ❑ Pencil, pen or permanent marker
- ❑ Labels
- ❑ Camera or smartphone

In the field or in the laboratory

1- Wear gloves.
2- Add water (tap water or water from your Study Site) to the lower compartment of the filtering unit up to the level of the outlet used to make vacuum. This reduces the volume of air that the syringe will aspirate to create vacuum. This increases the degree of vacuum and, consequently, the filtering speed.
3- Assemble the filtering body on the lower compartment. The filtering body, on which the membrane will be put, is fixed on the lower compartment by using gentle pressure.
4- Wet the surface of the filtering body using deionized water.
5- Gently remove a membrane (which you have previously marked with cardinal points and centre) from its packaging, using tweezers previously wetted with deionized water.
6- Place the membrane on the wetted filtering body.
7- Let the membrane wet.
8- Assemble the tubing on one outlet on the lower compartment and one rubber stopper on the other outlet.

9- Apply vacuum to the lower compartment by using a syringe. This allows to gain better contact between the membrane and the filtering body.
10- Immediately before use, rinse with deionised water the upper compartment and the lid of the filtering unit.
11- Screw the upper compartment onto the filtering body.
12- Ensure there is a filter on one of the little tubes on the lid of the upper compartment, and three rubber stoppers on the three other tubes. Put the lid on the upper compartment.
13- Agitate the sample inside the sampling bottle to homogenise its contents.
14- Let sit for 30 seconds (to settle the denser components like sand). Evaluate the turbidity and colour of the sample and read the *note "Considerations in the preparation of samples for analysis" on the Protocol.*
15- Lift the lid of the filtering unit for the strict time necessary to add the sample (250 mL or less at any one time – *e.g.* 50 mL or 100 mL). If the volume of sample being filtered is less than 250 mL, take note of its volume.
16- Fill the upper compartment until the meniscus of the liquid reaches the desired volume mark.
17- Pull the piston on the syringe to create vacuum. NOTE: The syringe can fill with water or a mix of water and air. It's good.
18- Remove the syringe from the tube and eject the air-liquid mixture. Reconnect the syringe to the tube.
19- Repeat the procedure to apply vacuum repeatedly, until all water in the sample is removed.
20- If filtering more than 250 mL, once the first volume has been removed by emptying the bottom compartment, top up the remaining volume and repeat steps to empty the upper compartment.
21- Lift the lid of the filtering unit and add deionised water using a spray bottle, to rinse the sides of the upper compartment, collecting particles that had become possibly adhered to the walls.
22- Re-apply vacuum until all deionised water is removed.
23- At the end of the filtration of the chosen volume, unscrew the upper compartment.
24- Gently lift the membrane using tweezers.
25- Open a Petri dish and place the membrane in it. Close the Petri dish.
26- Place a label on the lid of the Petri dish, stating the sample code recorded on the sample bottle.
27- Rinse the filtering unit with deionised water and prepare it for the following sample.

<u>Considerations in the preparation of samples for analysis</u>
The following aspects are to be taken into account when selecting the volume of sample to filter:
- turbidity of the sample (due to particles in suspension)
- presence of objects of size greater than 5 mm.

<u>If your sample is turbid</u>:
We recommend that you filter less than 500 mL per membrane. In case of notable turbidity, it is recommended that no more than 100 mL are filtered at any one time, preferably starting with 50 mL, to reduce the possibility of false negatives (caused by excess debris depositing over target items) and to reduce filtering difficulties (caused by the filter becoming clogged). In case of doubt, we recommend to start by filtering a small volume (50 mL) and add 50 mL at a time. Filtration should not be difficult. In the case of such low volumes being filtered we recommend that you only prepare 1, maximum 2, membranes per sample bottle, to ensure that the time taken to analyse your samples will not be excessive. Thoroughly shaking/mixing the bottle before filtration will help you have a still-significant sample.

<u>If you have deposit at the bottom of the bottle</u>:
Ensure you have collected as much “clear liquid as possible” and filtered that on membranes. Separately, add some liquid to the bottom of the bottle and shake, then filter the lifted deposit on a bespoke membrane. Rinse the sides of the bottle and filter on the same membrane.

Microplastics in Surface Waters – Sample Preparation through Filtration using a Syringe and Check Valves

Field or Lab Guide

Task

Prepare samples for the analysis, avoiding contamination. Filtering immediately after sampling avoids the formation of possibly compact deposits at the bottom of the container. These, hard to remove, might cause the loss of useful sample and could negatively affect the measurement.

Necessary Materials

- ❑ *Microplastics – Surface Waters Data Sheet*
- ❑ Filtering unit for biology
- ❑ 4 rubber stoppers for filtering unit
- ❑ 1 syringe filter for filtering unit
- ❑ Sterile membranes diameter = 47 mm (pore size 0.45 µm), gridded, minimum two membranes per sample
- ❑ Syringe 50 or 60 mL
- ❑ Latex, silicone or PVC tubing
- ❑ 1 x three-way connector with 2 x one-way valves (check valves) to connect the tubes to the syringe and the filtration unit
- ❑ Latex or vinyl gloves
- ❑ Petri dishes diameter = 55 mm
- ❑ 1 L distilled or deionized water
- ❑ Spray bottle containing deionized water
- ❑ Tweezers to handle the membranes
- ❑ Samples
- ❑ Rigid clipboard
- ❑ Pencil, pen or permanent marker
- ❑ Labels
- ❑ Camera or smartphone

In the field or in the laboratory

1- Wear gloves.
2- Assemble the filtering body on the lower compartment. The filtering body, on which the membrane will be put, is fixed on the lower compartment by using gentle pressure.
3- Wet the surface of the filtering body using deionized water.
4- Gently remove a membrane (which you have previously marked with cardinal points and centre) from its packaging, using tweezers previously wetted with deionized water.
5- Place the membrane on the wetted filtering body.
6- Let the membrane wet.

7- Assemble the tubing on one outlet on the lower compartment and one rubber stopper on the other outlet.
8- Apply vacuum to the lower compartment by using a syringe (and the Check-Valves). This allows to gain better contact between the membrane and the filtering body.
9- Immediately before use, rinse with deionised water the upper compartment and the lid of the filtering unit.
10- Screw the upper compartment onto the filtering body.
11- Ensure there is a filter on one of the little tubes on the lid of the upper compartment, and three rubber stoppers on the three other tubes. Put the lid on the upper compartment.
12- Agitate the sample inside the sampling bottle to homogenise its contents.
13- Let sit for 30 seconds (to settle the denser components like sand). Evaluate the turbidity and colour of the sample and read the note "*Considerations in the preparation of samples for analysis*" on the Protocol. If the sample appears turbid or "coloured" it is recommended that you filter the volume on a series of membranes, dividing the sample on across at least two membranes.
14- Lift the lid of the filtering unit for the strict time necessary to add the sample (250 mL or less at any one time – *e.g.* 50 mL or 100 mL). If the volume of sample being filtered is less than 250 mL, take note of its volume.
15- Fill the upper compartment until the meniscus of the liquid reaches the desired volume mark.
16- Pull the piston on the syringe to create vacuum. NOTE: the syringe must not fill with liquid: only air
17- Push and pull the piston of the syringe continuously, discarding the volume removed on the ground or in the bucket, until you have filtered the sample volume (up to 250 mL).
18- Continue to apply vacuum following this procedure, until all water in the sample is removed.
19- If filtering more than 250 mL, once the first volume has been removed by emptying the bottom compartment, top up the remaining volume and repeat steps to empty the upper compartment.
20- Lift the lid of the filtering unit and add deionised water using a spray bottle, to rinse the sides of the upper compartment, collecting particles that had become possibly adhered to the walls.
21- Re-apply vacuum until all deionised water is removed.
22- At the end of the filtration of the chosen volume, unscrew the upper compartment.
23- Gently lift the membrane using tweezers.
24- Open a Petri dish and place the membrane in it. Close the Petri dish.
25- Place a label on the lid of the Petri dish, stating the sample code recorded on the sample bottle.
26- Rinse the filtering unit with deionised water and prepare it for the following sample.

<u>Considerations in the preparation of samples for analysis</u>
The following aspects are to be taken into account when selecting the volume of sample to filter:
- turbidity of the sample (due to particles in suspension)
- presence of objects of size greater than 5 mm.

<u>If your sample is turbid</u>:
We recommend that you filter less than 500 mL per membrane. In case of notable turbidity, it is recommended that no more than 100 mL are filtered at any one time, preferably starting with 50 mL, to reduce the possibility of false negatives (caused by excess debris depositing over target items) and to reduce filtering difficulties (caused by the filter becoming clogged). In case of doubt, we recommend to start by filtering a small volume (50 mL) and add 50 mL at a time. Filtration should not be difficult. In the case of such low volumes being filtered we recommend that you only prepare 1, maximum 2, membranes per sample bottle, to ensure that the time taken to analyse your samples will not be excessive. Thoroughly shaking/mixing the bottle before filtration will help you have a still-significant sample.

<u>If you have deposit at the bottom of the bottle</u>:
Ensure you have collected as much "clear liquid as possible" and filtered that on membranes. Separately, add some liquid to the bottom of the bottle and shake, then filter the lifted deposit on a bespoke membrane. Rinse the sides of the bottle and filter on the same membrane.

Microplastics in Surface Waters – Sample Preparation through Filtration with Vacuum Line

Laboratory Guide

Task

Prepare samples for the analysis, avoiding contamination. Filtering immediately after sampling avoids the formation of possibly compact deposits at the bottom of the container. These, hard to remove, might cause the loss of useful sample and could negatively affect the measurement.

Necessary Materials

- ❑ *Microplastics – Surface Waters Data Sheet*
- ❑ Filtering unit for biology
- ❑ 4 rubber stoppers for filtering unit
- ❑ 1 syringe filter for filtering unit
- ❑ Sterile membranes diameter = 47 mm (pore size 0.45 µm), gridded, minimum two membranes per sample
- ❑ Vacuum line or vacuum pump and suitable tubing
- ❑ Latex, silicone or PVC tubing
- ❑ Latex or vinyl gloves
- ❑ Petri dishes diameter = 55 mm
- ❑ 1 L distilled or deionized water
- ❑ Spray bottle containing deionized water
- ❑ Tweezers to handle the membranes
- ❑ Samples
- ❑ Rigid clipboard
- ❑ Pencil, pen or permanent marker
- ❑ Labels
- ❑ Camera or smartphone

In the Laboratory

1- Wear gloves.
2- Assemble the filtering body on the lower compartment. The filtering body, on which the membrane will be put, is fixed on the lower compartment by using gentle pressure.
3- Wet the surface of the filtering body using deionized water.
4- Gently remove a membrane (which you have previously marked with cardinal points and centre) from its packaging, using tweezers previously wetted with deionized water.
5- Place the membrane on the wetted filtering body.
6- Let the membrane wet.
7- Assemble the tubing on one outlet on the lower compartment and one rubber stopper on the other outlet.
8- Apply vacuum to the lower compartment. Release the vacuum by gently removing the rubber stopper on the lower compartment and hearing the vacuum be released. Place the rubber stopper back on the lower compartment.

9- Immediately before use, rinse with deionised water the upper compartment and the lid of the filtering unit.
10- Screw the upper compartment onto the filtering body.
11- Ensure there is a filter on one of the little tubes on the lid of the upper compartment, and three rubber stoppers on the three other tubes. Put the lid on the upper compartment.
12- Agitate the sample inside the sampling bottle to homogenise its contents.
13- Let sit for 30 seconds (to settle the denser components like sand). Evaluate the turbidity and colour of the sample and read the note "*Considerations in the preparation of samples for analysis*" on the Protocol. If the sample appears turbid or "coloured" it is recommended that you filter the volume on a series of membranes, dividing the sample on across at least two membranes.
14- Lift the lid of the filtering unit for the strict time necessary to add the sample (250 mL or less at any one time – *e.g.* 50 mL or 100 mL). If the volume of sample being filtered is less than 250 mL, take note of its volume.
15- Fill the upper compartment until the meniscus of the liquid reaches the desired volume mark.
16- Apply vacuum.
17- If you are filtering more than 250 mL, after 250 mL are filtered, stop the vacuum application by closing the vacuum line tap or by stopping the vacuum pump and remove gently the rubber stopper which is located on the lower compartment. When you hear that the vacuum has equilibrated (hissing stops) you may proceed to lifting the filtering body and upper compartment from the lower compartment. Empty the lower compartment and re-assemble the device, paying attention to only adding the rubber stopper on the lower compartment as a last step.
18- Add the remaining volume into the upper compartment.
19- Filter the sample as described above.
20- Once the filtration of the sample volume is completed, stop the vacuum and release the vacuum gently removing the rubber stopper on the lower compartment.
21- Lift the filtering unit lid and with a spray bottle filled with deionised water rinse the sides of the filtering unit to remove particles which could have become attached to it.
22- Apply vacuum until all deionised water is removed
23- At the end of the filtration of the chosen volume, unscrew the upper compartment
24- Once you have completed the filtration and rinsing as above, stop the application of vacuum as described above and, once the pressure is equilibrated, undo the upper compartment by unscrewing it from the filtering body by turning it anticlockwise.
25- Gently lift the membrane using tweezers.
26- Open a Petri dish and place the membrane in it. Close the Petri dish.
27- Place a label on the Petri dish lid, with the sample code that was on the bottle and eventual information on the volume of sample that you filtered.
28- Rinse the filtering unit using deionised water and prepare it for filtering the remaining samples

<u>Considerations in the preparation of samples for analysis</u>

The following aspects are to be taken into account when selecting the volume of sample to filter:
- turbidity of the sample (due to particles in suspension)
- presence of objects of size greater than 5 mm.

<u>If your sample is turbid</u>:

We recommend that you filter less than 500 mL per membrane. In case of notable turbidity, it is recommended that no more than 100 mL are filtered at any one time, preferably starting with 50 mL, to reduce the possibility of false negatives (caused by excess debris depositing over target items) and to reduce filtering difficulties (caused by the filter becoming clogged). In case of doubt, we recommend to start by filtering a small volume (50 mL) and add 50 mL at a time. Filtration should not be difficult. In the case of such low volumes being filtered we recommend that you only prepare 1, maximum 2, membranes per sample bottle, to ensure that the time taken to analyse your samples will not be excessive. Thoroughly shaking/mixing the bottle before filtration will help you have a still-significant sample.

If you have deposit at the bottom of the bottle:
Ensure you have collected as much “clear liquid as possible” and filtered that on membranes. Separately, add some liquid to the bottom of the bottle and shake, then filter the lifted deposit on a bespoke membrane. Rinse the sides of the bottle and filter on the same membrane.

Microplastics in Surface Waters – Sample Observation through Microscopy

Field Guide

Task

Observe samples and analyse their content, avoiding possible contamination.

Necessary Materials

- ❑ *Microplastics – Surface Waters Data Sheet*
- ❑ *Microplastics Recognition Guide*
- ❑ Petri dishes containing the samples
- ❑ Latex or vinyl gloves
- ❑ Tweezers for handling membranes
- ❑ Optical microscope with light, better if with ocular calibrated measurement grid
- ❑ OPTIONAL: laptop for image collection if enabled on your microscope
- ❑ Camera or smartphone
- ❑ Glass slides to support the membrane
- ❑ OPTIONAL: calibrated measurement grid
- ❑ Pen or pencil
- ❑ Clipboard

In the field

1- Prepare the microscope on the lower magnification.
2- Using tweezers, take one sample from its Petri dish and place it in the middle of the glass slide (50 mm side) which you will place on the sample stage.
3- Rotate the membrane in the field of view, so as to see the lines on the membrane parallel and perpendicular to the observer, the centre point in the middle of the view, and such as to have N up, S down. This will facilitate recording of item coordinates and will facilitate navigation of the sample.
4- Close the Petri dish to prevent contamination.
5- Quickly scan the sample to quickly gain an understanding of its contents, using the printed grid (lines) on the membrane as reference system. Move on the sample following the grid, starting from the top-most point as follows: move from left to right, at the end of the run move one cell lower and move right to left, once at the end of the run move down by one cell and restart moving from left to right and proceed accordingly. You may take down the coordinates on the sample of objects you encounter and want to go back to later.
6- Once the first scan of the sample is concluded, you can restart the observation as described above, starting from the top-most point, and increasing the magnification as you wish to help with item identification.
7- Take note of your observations following the data sheet and using the reference images, the Microplastics Recognition Guide and datasheets for item recognition. Take photographs using a laptop or a smartphone for each item you observe that you wish to record as microplastic (remember, it's not just pieces of plastic that are microplastics, textile fibres are too!). Photos are necessary for data validation.
8- At the end of the observation, place the sample in its Petri dish and close the Petri dish for keeping.

9- Complete the summary datasheet.
10- On the Summary Datasheet that follows the table, you can calculate the TOTAL NUMBER OF PARTICLES FOUND PER CATEGORY
    a. If you have used more than one membrane to filter 500 mL, sum the data collected for each membrane to obtain the total number of particles per category present in the 500 mL sample.
    b. To obtain the total number of particles per category found in 1 m^3 of water, simply multiply by 2000 the particles you counted in every 500 ml sample.
11- Record the number obtained in your datasheet.
12- THE AVERAGE NUMBER OF PARTICLES FOUND PER CATEGORY
    Having collected three samples you can now calculate the average number of particles found in 1 m^3 of water, by averaging the values you have obtained for the three samples.
13- Record your observations in the Datasheet (Excel Spreadsheet) by adding necessary information and linking to each observation the photo file related to the object you are analysing. NOTE: some cells have multiple-choice menus for you to use.

Microplastics in Surface Waters – Sample Observation through Microscopy

Laboratory Guide

Task

Observe samples and analyse their content, avoiding possible contamination.

Necessary Materials

- ❑ *Microplastics – Surface Waters Data Sheet*
- ❑ *Microplastics Recognition Guide*
- ❑ Petri dishes containing the samples
- ❑ Latex or vinyl gloves (optional)
- ❑ Tweezers for handling membranes
- ❑ Optical microscope with light
- ❑ OPTIONAL: laptop
- ❑ Glass slides to support membranes
- ❑ OPTIONAL: calibrated measurement grid
- ❑ Pen or pencil
- ❑ Clipboard
- ❑ Camera or smartphone

In the laboratory

Note: If there is a fear of contaminating the sample, wear gloves

1- Prepare the microscope on the lower magnification.
2- Using tweezers, take one sample from its Petri dish and place it in the middle of the glass slide (50 mm side) which you will place on the sample stage.
3- Rotate the membrane in the field of view, so as to see the lines on the membrane parallel and perpendicular to the observer, the centre point in the middle of the view, and such as to have N up, S down. This will facilitate recording of item coordinates and will facilitate navigation of the sample.
4- Close the Petri dish to prevent contamination.
5- Quickly scan the sample to quickly gain an understanding of its contents, using the printed grid (lines) on the membrane as reference system. Move on the sample following the grid, starting from the top-most point as follows: move from left to right, at the end of the run move one cell lower and move right to left, once at the end of the run move down by one cell and restart moving from left to right and proceed accordingly. You may take down the coordinates on the sample of objects you encounter and want to go back to later.
6- Once the first scan of the sample is concluded, you can restart the observation as described above, starting from the top-most point, and increasing the magnification as you wish to help with item identification.
7- Take note of your observations following the data sheet and using the reference images, the Microplastics Recognition Guide and datasheets for item recognition. Take photographs using a laptop or a smartphone for each item you observe that you wish to record as microplastic (remember, it's not

just pieces of plastic that are microplastics, textile fibres are too!). Photos are necessary for data validation.

8- At the end of the observation, place the sample in its Petri dish and close the Petri dish for keeping.

9- Complete the summary datasheet.

10- On the Summary Datasheet that follows the table, you can calculate the TOTAL NUMBER OF PARTICLES FOUND PER CATEGORY

   a. If you have used more than one membrane to filter 500 mL, sum the data collected for each membrane to obtain the total number of particles per category present in the 500 mL sample.
   b. To obtain the total number of particles per category found in 1 m^3 of water, simply multiply by 2000 the particles you counted in every 500 ml sample.

11- Record the number obtained in your datasheet.

12- THE AVERAGE NUMBER OF PARTICLES FOUND PER CATEGORY
Having collected three samples you can now calculate the average number of particles found in 1 m^3 of water, by averaging the values you have obtained for the three samples.

13- Record your observations in the Datasheet (Excel Spreadsheet) by adding necessary information and linking to each observation the photo file related to the object you are analysing. NOTE: some cells have multiple-choice menus for you to use.

Frequently Asked Questions

1. Why do I have to collect more than one sample per site?

Microplastics are solid objects that are found dispersed in water bodies. Given their particular nature, the concentration of microplastics in large volumes of water can be not homogeneous. The more a body of water is stagnant, the easier it is for microplastics to accumulate in particular points. It's critical that you obtain a sample that is *representative* of the sampling site. It's therefore important that you collect three or more samples for each station, especially for those with stagnant or low speed waters. Measuring the concentration of microplastics across three or more samples also reduces the chances of the influence of potential contamination in your analysis.

2. What makes the concentration of microplastics change during the year?

Seasonal temperature differences cause density variations in surface waters and often cause the mixing of water volumes. This can cause microplastics to move in the body of water you are sampling, and cause concentration variations. Seasonal variations can also affect water flow in rivers, currents, waves and salinity, as well as winds and the effects of winds on surface waters (and debris they carry). All these factors may cause the redistribution of species suspended in surface waters, such as microplastics, and can cause their accumulation as well as their dilution.

3. Why/when do I have to divide the sample in several smaller volumes and filter them separately?

For "clean" waters, you should be able to filter 500 mL per membrane without problems. To judge whether you should be filtering smaller volumes, look at the turbidity of your sample. Water turbidity is due to small solids suspended in the water. The greater the turbidity, the more the solids that are suspended. You should filter a small volume if you have a "cloudy/turbid" or "coloured" sample for two reasons. Firstly, when you filter, all solids fall on top of each other in layers that are not transparent. Excessive amounts of solids can result in debris "hiding" microplastics, in your sample. Secondly, a sample with many solids can become difficult to filter in reasonable time. If filtration becomes slow at the end of the filtration of 250 mL, please use a second membrane for the second 500mL volume. We therefore ask you to discuss with your teacher whether you should filter the whole 500 mL using a single membrane.

4. If I have to divide the sample, how do I calculate the total number of microplastics in the original sample and in a standard volume of water?

Filtering a sample in stages consists in filtering a volume lower than 500 mL per membrane. Dividing your sample does not cause you to loose microplastics. If you divide a 500 mL sample in 250 mL volumes per membrane, from a 500 mL sample you would obtain 2 membranes. The number of microplastics present in the original 500 mL volume is calculated as the sum of all items found in the two membranes.

5. Why do we also take note of textile cellulose and textile animal fibres in the samples?

It is likely that the majority of the samples you collect present as a high percentage of textile microfibres, of which many of natural origin. Among these, the most recognisable are wool (scales perpendicular to the length) and cellulose fibres such as cotton (appearing like a flat ribbon). Even if these are fibres of natural origin, they are introduced by man into the hydrosphere.

Many fibres, including natural ones, are subjected to several processes which alter their chemical composition. Among the most common treatments, are those that deposit dyeing agents or coatings, the latter often made of polymers or plastics. It's important to measure the presence of all textile fibres, and to identify their likely composition (cellulose, animal, man-made-looking) as their presence is an index of pollution by textiles due to their use (loss of fibres during use). This in turn is an index of the presence of domestic or industrial wastewater contributions.

Natural fibres absorb large quantities of water, equal to, or some time greater than, their own dry weight. This gives natural fibres (especially cellulose-based fibres) next-to-neutral buoyancy. These are equally likely to be found throughout the water column. Man-made fibres often have a higher density than that of water, and they are often seen at the water surface due to interfacial tension and wettability effects (synthetic fibres are often hydrophobic), however, it is now becoming clear in the literature that their presence in the depths of a water body is greater than at its surface. These can therefore escape our measurements. Both synthetic and natural fibres are lost through daily use (e.g. at the beach, while riding, walking, rubbing fabrics together, etc.) and through wash-water discharge. Natural fibres can be considered a proxy, an indicator of the presence of other microfibers, which are harder to find in surface waters.

6. I am not certain that the object I am looking at is plastic. What do I need to do?

The appearance of microplastics can be deceptive. This is due to a number of factors. The main factor is plastic degradation due to atmospheric and fluid agents. Another cause of "changed" appearance is the growth of biofilm. This is facilitated by the hydrophobicity typical of most plastics. In specialised laboratories, samples are exposed to aggressive chemicals that eliminate the biological components, hence reducing the uncertainty in identification. While it might be that your school is able to allow you to run such preparation following the NOAA guidelines (https://marinedebris.noaa.gov/sites/default/files/publications-files/noaa_microplastics_methods_manual.pdf), this Protocol intends to guide you to observing the samples as they are without chemical processing. If you are unsure of what you are looking at, take note of other features of the object: colour, dimensions, shape. Note if the object is porous, shiny, opaque, whether it has scratches, etc. Mark the object as unknown in your Data Sheet. Take a photo and load it with your data.

All the information you gather on each particle can help you to identify biological or natural-occurring residue from microplastics. The more you will look at samples, the more advanced your ability to positively identify microplastics will become. Try to observe your sample under a stereo microscope at school, if you have only looked at it in the field. We also recommend you contact a University, a research centre or an environmental protection or monitoring agency to whom you can pass the samples for confirmation of your observations. Alternatively, if you can, take a photo and share with an expert for their input.

7. I have found object of very regular shape or pattern. Are these microplastics?

The appearance of biological objects in marine as well as fresh waters can be deceitful. You might make some interesting biological encounters: tubular or needle-like structures, small round particles, sometimes with dimples resembling a transparent golf-ball, and "glassy stars", geometric structures made up of monocellular algae. These are very common encounters in surface waters.

The splendid geometry of these shapes, with such a fine level of control, is a signal that these shapes are most likely of biological origin.

On the other hand, you may come across microfibres that show channels or ridges. These are likely the only artificial structures that humankind can manufacture at scale with high control on such regular microscopic morphology. The Microplastics Recognition Guide should help you recognise these as plastics. If you are in doubt after discussing with your teacher, take a photo and share it with an expert.

8. How likely am I to find white plastics?

The appearance of many particles of natural or even synthetic origin can be deceiving. This is the case for "white", opaque and transparent particles. Literature reports state that depending on your location you may find objects such as paraffin wax particles. These are not plastic and would crumble if you touched them. If you are unsure, record these particles as "unknown" and seek help from your teacher. Always take a photo and load it with your data.

9. Why do I need to run a blank or control sample?

When we measure microplastics in the environment, we use tools and procedures that can contaminate our samples. This means that what we do could be adding microplastics to the water sample, therefore giving us data that is not representative of the true concentration of microplastics in the water sample. Contamination is something we can reduce, but it is very difficult to eliminate completely. That's why we measure it.

Running a blank or control sample allows us to understand the potential for contamination. We take this into account when we look at the data, so that we subtract the likely process-driven contamination that is very hard to avoid.

You can discuss with your teacher how preparing a blank or control sample in your laboratory can also tell you about the presence of microplastics in the air in your school.

10. How do I prepare a blank?

You can prepare "blanks" or "control samples" in different ways, as mentioned in the Teachers' Guide. .

Examples of blanks:

- A new membrane left on the microscope stage for set periods of time (1-3 h) to observe airborne contamination;
- A filtered deionised water sample;
- A sample prepared using deionised water but without applying the air filter on the lid for the filtration unit;
- A sample prepared by filtering a volume of water which you had left in a beaker on a cupboard at home or at school for a period of time;
- A new membrane let sit in a spot in the classroom over the weekend.

Observing and measuring the extent of contamination that is possible due to airborne (and not only) microplastics is a bonus aspect of this protocol. By measuring airborne or water-borne contamination in your laboratory, you are generating data no one else has previously generated, which might inform atmospheric (indoors and outdoors) studies in the future.

11. Can I sample snow or ice?

Absolutely. We welcome you to test whichever form of water you are interested in. In the case of ice and snow, record the weight of the sample: 500 g = 0.5 L.

Microplastics – Surface Waters

Paper Data Sheet – complete one per sample

Name of your School: _____ Class or team name: _____
Student/s name/s: _____
Teacher/s name/s: _____

Sampling date: Year: _____ Month: _____ Day: _____ Time (24h): _____
Study Site: _____
Study Site Latitude (XX°YY' N or XXX,ZZZ° S): Longitude (XX°YY' N or XXX,ZZZ° S):
Sample code: _____
Temperature: _____°C
Total volume filtered: _____

Filtration
How many membranes did you use to filter this sample? _____
Filter Membrane 1 Volume: _____
Filter Membrane 2 Volume: _____
Filter Membrane 3 Volume: _____

Imaging and Observation
Microscope model: _____
Light Source: _____

Note below observations about your Study Site and your sampling experience. Report: visible sources of plastic pollution (bins, landfill, etc.) at the site, any visible plastic debris floating on the surface, any visible plastic debris on the shores of your Study Site. NOTE: if you notice plastic debris that you believe do not belong in the environment, please notify your teacher and discuss options for the removal or notification of local Government. You might want your teacher to retain these for reference, as you may be able to relate them to what you will find in your sample. Attach photos or drawings, if you wish.

_____

_____

Now identify the objects you recognise as microplastics, textile animal fibres, textile cellulose fibres, textile man-made fibres and unknown objects (suspicious, could be microplastics) by using the following table, adding as much information as possible. Each object will be described in one row. At the end of the process of noting all particles, count them and report them in the table that follows.

NOTE: If you are taking images (what we highly recommend) name them following this convention: NumberofItem_magnification.jpg - e.*g. 1_100x.jpg* *(please, see pages 40-41)*

DATASHEET

DATASHEET

SAMPLE CODE: _____

Table 1. *Number and characteristics of relevant objects identified through observation under a microscope. Print more sheets and add numbers if you need. You will need to put this information in the Datasheet provided.*

Membrane Number	***Object number***	***<u>Geometry</u>*** ***- Fibre/filament*** ***- Round particle*** ***- Flat particle or sheet.***	***<u>Colour</u>***	***<u>Surface appearance</u>*** ***- Shiny*** ***- Rough or porous,*** ***- Scales***	***Longest dimension (µm) (estimate) OPTIONAL***	***Shortest dimension (µm) (estimate) OPTIONAL***	***File name for the photo*** ***Photo number with link to the photo***	***<u>Your assessment:</u>*** • ***Plastic piece*** • ***Textile cellulose fibre,*** • ***Man-made textile fibre*** • ***Textile animal fibre*** • ***Unknown***	***<u>Membrane Coordinates</u>*** ***(N, S, E, W and step count)***

DATASHEET

Example of compiled Datasheet

You have collected three water samples, 500 mL each, in the Sample Site "SAVA River Gardens".
The Sample Codes of the three samples are: SAVA RD 1, SAVA RD 2, SAVA RD 3.
You have filtered the sample SAVA RD 1, dividing the 500 mL sample in three aliquots, respectively filtered on the membrane 1 (200 mL), membrane 2 (150 mL) and membrane 3 (150 mL).
You have found and classified 3 objects on membrane 1, 5 objects on membrane 2 and 4 objects on membrane 3.

Membrane number	*Object number*	***Geometry*** ***- Fibre/filament*** ***- Round particle*** ***- Flat particle or sheet.***	***Colour***	***Surface appearance*** ***- Shiny*** ***- Rough or porous,*** ***- Scales***	***Longest dimension (estimated - µm)*** ***OPTIONAL***	***Shortest dimension (estimated - µm)*** ***OPTIONAL***	***File name for the photo (or link)*** ***Photo number and magnification with link to the photo***	***Your assessment:*** • ***Plastic piece*** • ***Textile cellulose fibre,*** • ***Man-made textile fibre*** • ***Textile animal fibre*** • ***Unknown***	***Membrane Coordinates*** ***(N, S, E, W and step count)***
1 (200mL)	1	fibre	yellow	rough	350	20	1_40x	Textile cellulose fibre	N5W2
	2						2_40x		
	3						3_100x		
2 (150 m)	4						4_400x		
	5						5_40x		
	6						6_40x		
	7						7_400x		
	8						8_100x		
3 (150 mL)	9						9_40x		
	10						10_100x		
	11						11_40x		
	12						12_400x		

Where and how to save photos and Datasheets and link the Datasheet to the photos

- Before observing the membrane under a microscope, create a folder in the PC in use with the name of the sample to be analysed, eg. SAVA RG 1
- As you locate an item, fill in the row of the SAVA RG 1 sample **paper Datasheet** and take the photo of the object; save the photo in the folder with **the progressive number, followed by the magnification of the photo**. Remember: the total magnification is the product of eyepiece magnification by the objective magnification, *e.g.* 3-40x (photo of the object number 3, with magnification 40x)
- After reading the three membranes under the microscope, fill in the general section of the **Digital Datasheet** and save it in the SAVA RG 1 folder, with the same name.
- Fill in the specific part relating to the objects.
- In the column *Photo Number and magnification with Link to* the photo, for each object create the link to the respective photo.
- Save the folder.

Note: in this way teachers can verify the correctness or otherwise of the classification and possibly modify it if incorrect. The magnification reported in the file name helps the teachers verify dimensions, if you have used a calibrated microscope ruler and you have reference images for that. In other words, the link to the photos allows teachers to validate the results and build a reliable Database.

SUMMARY DATASHEET

Your findings (fill in at the end of the observation for each sample)

Report below how many items you have found for each category: plastic fragments, textile cellulose fibres, textile animal fibres, man-made textile fibres, unknown (suspicious objects which could be microplastics).

Volume of water filtered (mL): _____

	Particles found in the volume filtered	****Equivalent number of particles per m³***
Plastic fragments		
Textile cellulose + animal fibres		
Man-made fibres		
Unknown (suspicious objects which could be microplastics)		

To find out the number of particles per m^3, you need to use proportions. 1 m^3 contains 1000L. The variables in red below are the ones you have.

Volume you filtered in L / 1000 L = number of particles you found / **number of particles per m^3**

This can be written as:

$$\textbf{Number of particles per m}^3 = \frac{1000\ \text{L} * (\text{Number of particles you found})}{\text{Volume you filtered (L)}}$$

Example

If you have filtered 500 mL (which is 0.5 L), and found 12 plastic fragments, you can use the formula above to calculate how many particles that would equate to in 1 m^3:

12 particles x (1000 L /0.5 L) = 12 particles x 2000 = **24000 particles in 1 m^3**

Which is a lot!!! …but remember – this is a very rough approximation!

END OF DATASHEET

Microplastics Protocol – Reflecting on the data

Is the data reasonable?

The occurrence of microplastics in the environment can vary as a function of atmospheric conditions (at time of sampling and up to a few days before) and human activity.

Many factors play a role in the concentration of microplastics in surface waters. The movement of waves or mechanical action due to wind or the passage of animals or vessels can also contribute to the resurfacing of plastics that might have been lying in the depth of the water column. Salinity and water temperature also affect the density of water and changes in temperature might cause redistribution of microplastics during the year. Storm water runoff is another factor that can affect what you will find in a sample. The effect is hard to predict. Rain might wash plastic debris from roads, paths and ground around your study site and you might encounter a higher concentration after rain than you would at other times. Rain might also cause the dilution of microplastics in your site, with the consequence that you may find lower concentrations of microplastics after the rain.

Ideally, you will not find any particles that you can identify as microplastics. Typically, though, and depending on local conditions, you might come across 1-10 particles in a 500 mL sample, that you might clearly identify as microplastics (including textile fibres of all types). This corresponds to a value of respectively 2,000 to 20,000 particles per cubic metre. It's, remember, a very rough estimate.

You should pay attention to the type and colour of particles you observe. You might be able to relate them to larger plastic debris present in your study site. You should also note there is a potential for contamination from items you used for sampling or during sample handling. You should prepare "control samples" or "blanks" to understand the chance of contamination during processing. You can also set up a blank sample next to the microscopes for a period of time and then observe it. This would give you an idea of the level of atmospheric contamination that could affect your sample in your laboratory. It's unlikely that the observation stage of the samples will lead to severe contamination, unless you are not being careful or taking a long time, but it might.

What do we look for in the data?

The presence of microplastics in surface waters can have serious effects on the ecosystem both of physical and chemical nature. The presence of microplastics in water is a sign of pollution and poor water quality. It's important to add microplastics data to the other indices that are routinely monitored in order to establish whether there are correlations with whole-of-hydrosphere health. Microplastics don't belong in the environment. As such, they are alien species that can have significant biological effects. They can accumulate noxious chemicals on their surface, as well as provide fertile ground for the formation of pathogen-ridden biofilms favouring their presence where they would not otherwise thrive.

While microplastics are not monitored with the aim of removing them from aqueous environments (too hard a task at present), it is important to monitor their presence to see if our present and future interventions are having the desired effects. Additionally, it's important to monitor microplastics because they are considered an indicator of pollution,and your measurements may discover previously unknown sources of pollution, which you can communicate to environmental protection and monitoring agencies.

Construction of filtration system with check-valves