SAMPLE PREPARATION MANUAL

FOR THE ANALYSIS OF PLASTIC-RELATED POLLUTANTS

EDITED BY AMAREIN J FOURIE AND DANICA MARLIN – **2022 EDITION**

TOWARDS ZERO PLASTICS TO THE SEAS OF AFRICA

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Disclaimer
The guidance and recommendations provided in this manual are intended for competent users operating within the norms and laws of their respective countries. SST does not accept liability resulting from the use of the sample preparation manual.

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PREFACE

The occurrence of plastics in the natural environment has been reported from the most remote places on earth. As such, there is growing global concern about the potential human health impacts caused by plastic pollution and an urgency to implement effective actions to mitigate such pollution. International organisations have, therefore, called for the standardisation of methods for collecting data on plastic pollution. For example, in March 2019, UNEA noted the "need for high-quality data and effective monitoring… of marine litter, including plastic litter and microplastics, …through harmonised methodologies, to enable better and more effective action."

Information about microplastic particles in the environment, be it in water, air, sediment, or organisms, is being collected by many researchers, increasing our knowledge on the subject, and methods for collecting microplastic particles already exist. A good example is the guidelines for monitoring and assessing plastic litter and microplastics, written by the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP). Information on emerging contaminants associated with microplastics, such as bisphenols and benzophenone UV-filters, is also being collected by researchers. However, to the best of my knowledge, there are no guidelines for the very first step in monitoring emerging contaminants – that is, the preparation of samples before they get analysed for these plastic-related contaminants, i.e. pollutants. This manual was, therefore, produced for two reasons. Firstly, to answer the call to harmonise methods, and secondly, to provide guidelines for preparing samples to be ready for analysis for emerging contaminants.

This manual is aimed at African researchers with limited resources who may have English as a second language, if not third or fourth. It is, therefore, purposefully written in as simple language as possible, with many photographs showing all the steps to prepare samples for analysis carefully. The methods are set out clearly, in an orderly fashion, so that they can be followed by researchers who may not be familiar with sample preparation. It is hoped that the manual will become the standard guideline for researchers to use in their investigations of plastic-associated pollutants. This will not only improve our understanding of potential human health impacts resulting from plastic pollution but will also aid in filling the data gap that exists for this line of research in Africa.

Dr Danica Marlin

Head of Research at Sustainable Seas Trust

CHAPTER 1
PLASTICS AND THEIR ASSOCIATED POLLUTANTS

1.1. PLASTIC LITTER SIZES AND MICROPLASTICS

Marine litter is defined as any persistent solid material, manufactured or processed, that is discarded, disposed of, or abandoned in the marine and coastal environment[1]. Marine plastic litter items can be grouped into four main size classes:

- microplastics
- mesoplastics
- macroplastics
- megaplastics

as summarised in **Table 1.1.**

For the purpose of this manual, "plastic" refers to all sizes of plastics, but the focus will be on microplastics. This will be explained in the sections below.

Microplastics are small plastic particles consisting of fragments, fibres, or granules. Microplastics are commonly defined as plastic particles less than 5 mm in size [2-4]. There are two main groups of microplastics (**Figure 1.1**). Primary microplastics are plastics intentionally manufactured to be small in size and include items such as plastic pellets, which serve as raw plastic materials, and microbeads, commonly used in facial cleansers and cosmetics [2,5,6].

Secondary microplastics originate from the fragmentation (the process of being broken into smaller pieces) of larger plastic items (like meso-, macro-, and megaplastics) in the environment through various biological and chemical processes [2,7-9]. This group of microplastic particles contributes to the majority of microplastics in the environment due to the large amount of larger plastic litter items found in the environment [7,10].

Plastics, and therefore microplastics, have the potential to persist in the marine environment for a very long time due to their slow degradation processes[10-12]. This raises concern about the harmful effects plastic may have on marine organisms and the consequent impacts on human health[2,13-15].

Table 1.1: Summary of size definitions of marine plastic litter and common sources, adapted from GESAMP 2016 [4].

Size categories of marine plastic litter	Diameter			
	Micro <5mm	**Meso <2.5cm**	**Macro <1 m**	**Mega >1 m**
Source	*Primary microplastics.* *Secondary microplastics: fragmentation of larger plastic items.*	*Direct and indirect*: including fragmentation of larger plastic items.*	*Direct*: lost items from maritime activities or rivers.*	*Direct: abandoned fishing gear, catastrophic events.*
Examples of marine litter	*Primary: resin beads, microbeads from personal care products. Secondary: textile fibres, tyre dust.*	*Bottle caps, fragments.*	*Plastic bags, food and other packaging, fishing floats, buoys, balloons.*	*Abandoned fishing nets and traps, rope, boat hulls, plastic films from agriculture.*

** Direct: meaning the litter source is a primary plastic product fitting the size range. Indirect: meaning the litter source is the fragmentation of plastic items.*

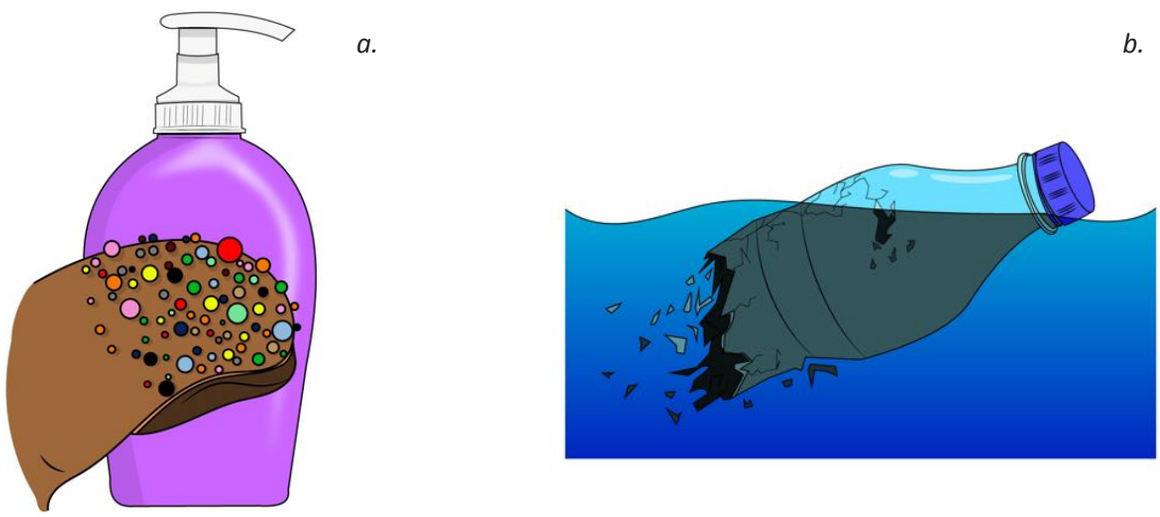


Figure 1.1: Examples of a) primary microplastics, such as microbeads used in personal care products, and b) secondary microplastics, such as those formed by a fragmenting plastic bottle in the ocean.

1.2. PLASTIC-RELATED POLLUTANTS

All plastics, regardless of their size, can leach (leak), absorb (soak up), and adsorb (adhere to their surface) pollutants to and from the environment (see **Figure 1.2** for explanations of these terms).

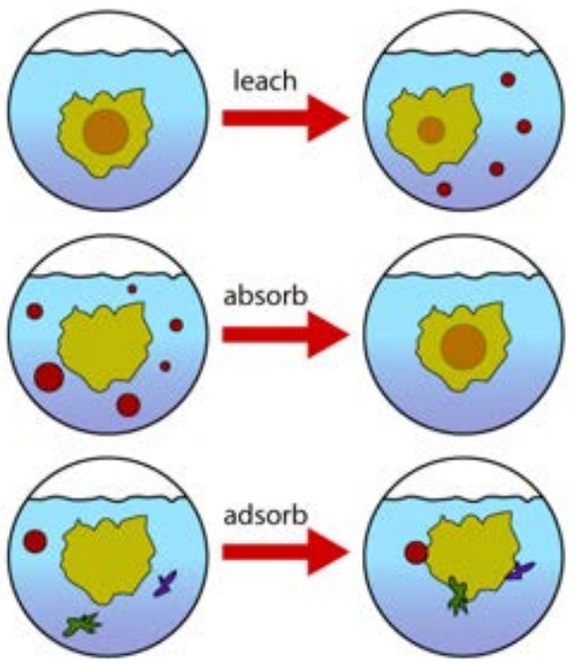


Figure 1.2: Microplastics leach, absorb, and adsorb plastic-related pollutants from the water column.

A complex mixture of chemicals may be associated with plastics. These include chemicals used in the production process of plastics and are thus intrinsic constituents (the essential ingredients) of plastics. Plastics are produced by combining monomers (the building blocks of plastics), which are molecules such as ethylene and propylene. When combined, multiple monomers form polymers, such as polyethylene and polypropylene.

Various chemicals are used during this process, including solvents and chemicals that act as catalysts (a substance that aids the chemical reaction). Additives are then added to give plastic its specific characteristics, such as flexibility, strength, colour, and UV resistance. These additives can include UV stabilisers, fillers, pigments, phthalates, 4-nonylphenol, and flame retardants[16-18]. Several of these chemicals are known to present toxic risks to plants, animals, and humans. Bisphenol-A (BPA), for example, is one of the monomers used in the production of plastics. However, it is also a known endocrine disruptor because it mimics the human hormone oestrogen [2]. Styrene and polyvinyl chloride can be carcinogenic or mutagenic, which can result in cancer or gene mutations[2,19,20].

Additionally, chemicals in the water surrounding the plastics may adsorb onto the surface of plastics in aquatic ecosystems due to their hydrophobicity (water-fearing properties). These chemicals include a variety of persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), polycyclic aromatic hydrocarbons (PAH), organochlorine pesticides (OCPs), brominated or fluorinated flame retardants, and perfluoroalkyl acids, among others. Metals also adsorb onto microplastics. Therefore, (micro)plastics may act as a vector for hazardous pollutants, exposing marine organisms to such pollutants when plastics are unintentionally ingested[7,21].

It is important to note that chemicals that adsorb onto plastics, and in fact also many of the chemicals that are intrinsic to plastics, have numerous anthropogenic sources. These sources include domestic and industrial wastewater as well as other consumer products that are lined with plastics, such as aluminium cans. In many cases, this makes it difficult to conclude that plastics alone are the source of the chemicals.

1.3. ENVIRONMENTAL AND HUMAN HEALTH RISKS

Due to their small size and abundance in aquatic environments, microplastics are easily accessible to a variety of organisms in the food web[2], including riverine and marine invertebrates, fish, turtles, seabirds, and marine mammals [8,13,22].

Filter-feeding invertebrates in the lower trophic levels of the food web inadvertently consume these small particles as they are non-selective feeders (**Figure 1.3**).

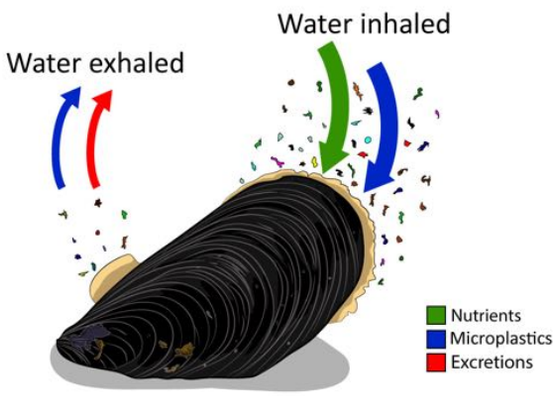


Figure 1.3: Non-selective feeding by filter feeders allows ingestion of microplastics.

Microplastic ingestion can occur directly, i.e., through accidental consumption of microplastics, or indirectly, when predators consume prey contaminated with microplastics in lower trophic levels [23,24]. Numerous fish species have also been recorded to ingest meso- and macroplastics along with microplastics [22].

Furthermore, the potential also exists for the bioaccumulation (build-up within a single organism over its lifespan) and biomagnification (transfer from lower to higher trophic levels) of plastics and their related pollutants in the food web [13,25]. Consequently, (micro)plastics and their related pollutants may contaminate aquatic food webs. This contamination makes (micro)plastics a potential risk to human health, as they have been detected in seafood such as mussels, oysters, and fish that is sold for human consumption [13,26-28].

CHAPTER 2
GENERAL PROTOCOLS

2.1. THE PURPOSE OF THE SAMPLE PREPARATION MANUAL

Limited information exists on plastic-related pollutants in marine organisms from Africa, while there is a need for short- and long-term monitoring of these pollutants in the environment.

This manual aims to provide harmonised methods for preparing samples for the analysis of plastic-related pollutants to build baseline data for Africa. We recognise that there are several methods available to investigate the presence of pollutants in organisms and the environment. However, there is a need for harmonised methods to carry out plastic-related pollutant research.

This manual aims to provide the simplest and least expensive methods used to prepare samples, with enough detail to allow for replication, so that the methods are feasible for developing countries, such as those in Africa. However, pollutant research requires specialised equipment.

Therefore, we encourage collaborations between researchers, laboratories, research facilities, and universities to facilitate this research. These collaborations would ensure that data will be comparable between studies in different areas and that there will be a collective and collaborative effort to contribute to and build on the limited published literature in Africa on plastic-related pollutants in marine organisms.

Detailed methods are described in this manual on the sample preparation process that needs to take place before samples can be analysed for potentially harmful trace elements (PHTEs) and common plastics constituents (CPCs), collectively termed plastic-related pollutants, in organisms (specifically those commonly consumed by humans, such as bivalves and fish). PHTEs, as defined in this manual, include heavy metals, non-metals, and rare earth elements. CPCs include plastic polymers such as bisphenol analogues and plastic additives, such as benzophenone-ultraviolet (BP-UV) filters.

The objective of this manual is to implement harmonised methods across Africa to allow for data sharing and comparison across countries to build baselines and initiate long-term monitoring programmes. This manual will enable researchers to prepare samples to investigate these pollutants in a particular environment and organism(s).

This manual does not include methods to investigate (micro)plastic particles in organisms. Standard operating procedures (SOPs) to investigate microplastic particles in organisms, water, and sediment are available from the Commonwealth Litter Programme (CLiP), led by the UK through the Centre for Environment Fisheries and Aquaculture Science (Cefas).

Please feel free to send your request to info@sst.org.za to receive a copy of the SOPs.

2.2. POTENTIAL RESEARCH QUESTIONS

Ensure that you have formulated your research questions based on what you would like to answer through your research before using the manual. For example:

- What are the concentrations of PHTEs and CPCs in bivalves/fish/from your specific study area?
- Is there a difference in the concentrations of PHTEs and CPCs between different species or sites?
- Can specific pollution sources be detected by investigating the concentrations of PHTEs and CPCs in bivalves/fish?
- Are there seasonal differences in the concentrations of PHTEs and CPCs?

2.3. IMPORTANT RECOMMENDATIONS

Contamination is an important risk factor to keep in mind during all the steps of the preparation method. More detail is provided in each chapter, but below is a quick summary:

- Ensure you have a clean work area and equipment.
- Ensure the right equipment is used for the different sample analysis types.
- Aim for a speedy process. Planning is essential to ensure that there is enough time to process the samples on the same day as sample collection to avoid contamination. There are four main steps: laboratory and equipment preparation, sample collection, sample preparation, and freeze-drying.
- Ensure each sample is homogenised (mixed) thoroughly to avoid high data variability as a result of differences in concentrations within the tissues of a sample.

2.4. SAFETY AND STUDY REQUIREMENTS

Please take careful note of the following safety aspects as well as special requirements for sampling:

- Always consider the safety of individuals when going out sampling. If samples need to be collected in remote areas, ensure that a minimum of three people goes out to the study site.
- Safety items to take into the field may include life vests, flashlights, pepper spray, a hat, drinking water, and reflective clothing.
- Ensure you have the necessary permits for sampling in riverine and marine environments. **Permits** will usually require that you name the organisms and/or area that you wish to sample. Permitting procedures will differ between countries, but permits are needed for publication purposes and to ensure no harm was done to the environment and/or other species during sampling.
- Ensure you have **ethics** approval where necessary. For example, research on fish requires ethics approval to ensure the fish are handled correctly and euthanised humanely.
- Look at the weather forecast and tidal cycle before planning the sampling trip.
- The preparation and subsequent analysis of samples require specialised equipment and expertise (please refer to Chapter 3). Therefore, it is important to consult with the technician at the laboratory or facility that you will use to ensure the correct equipment is available to proceed.

CHAPTER 3

BIOLOGICAL SAMPLE COLLECTION AND PREPARATION FOR THE ANALYSIS OF PLASTIC-RELATED POLLUTANTS

3.1. OVERVIEW

Plastics and plastic-related pollutants have many harmful effects on marine and riverine organisms, but there are many uncertainties regarding their potential risk to human health if consumed. But first the concentations of these pollutants need to be determined. Bivalves such as mussels and oysters are commonly used as bioindicators to monitor pollutant levels of PHTEs, CPCs, and other contaminants in marine and riverine systems[24,29,36]. These organisms are sedentary filter feeders and are thus susceptible to accumulating plastic-related pollutants in their tissues, thereby reflecting the magnitude of contamination in their surrounding environment [33,34,37,38].

Mussels and oysters are also common mariculture species and are of particular concern because elevated pollutant levels in these species may pose a risk to human health when consumed [33,37]. Fish is another important food source for humans, and many communities rely on fish for their livelihoods.

Some marine fish species, such as tuna, may accumulate PHTE, particularly mercury, in high concentrations [39]. Therefore, it is important to regularly monitor pollutants in aquatic environments, especially in species often consumed by humans.

3.1.1 OBJECTIVES

This chapter aims to provide the method and equipment needed for **sample preparation** for the analysis of potentially harmful trace elements (PHTEs) and common plastics constituents (CPCs) in biological samples that are intrinsic to or adsorbed onto (micro)plastics. Literature shows that seafood consumed by humans is contaminated with plastic-related pollutants, but these studies have mainly been conducted outside of Africa [40].

Studies have taken place in Europe [24], Indonesia, and the USA [27], while Africa is data-poor with regards to the extent of potential seafood contamination.

This highlights the importance of research into plastic-related pollutants in organisms and the value it could bring to scientific and public communities.

3.2. SPECIES

Focus on species that are an important food source for humans. Ideally, organisms found at two trophic levels of the food web should be sampled: invertebrates (generally found on the bottom of the food web) and fish (generally found at the mid and top level of the food web). Again, ensure that you have the correct permits to sample your selected species.

The following organisms can be used as a starting point to determine plastic-related pollutants in food webs:

a) Invertebrates: bivalves, such as oysters and/or mussels, commonly consumed by humans.

b) Fish: focus on species (and size of the fish) important for commercial and recreational use in the study area. Smaller fish species that are common prey items to the fish species often consumed by humans can also be sampled.

3.3. SAMPLE PREPARATION METHODS

3.3.1. BIVALVES

3.3.1.1. OVERVIEW AND MATERIALS

Below is a detailed, step-by-step description of methods for the preparation of samples, from sample collection to the freeze-drying process, specifically for mussels or oysters, to be analysed for PHTEs and/or CPCs using mass spectrometry (section 3.4). Since humans consume the whole organism in the case of mussels and oysters, a single sample consists of the entire organism. The methods below describe the collection of organisms that are easily accessible, e.g., mussels on the rocky shore, without requiring the need to scuba-dive or snorkel. Should diving be required, abide by all regulations pertaining to diving and all national legislation where necessary.

Take careful note of the **type of material** the equipment is made from to handle samples for the analysis of different pollutant types. **Table 3.1** summarises the equipment material for the analysis of each pollutant type. Keep in mind that the equipment material type in the photos used is for illustrative purposes ***only***; please confirm the material type you need to use based on the table below.

For the analysis of **PHTEs**, all the equipment (knives, forceps, spatulas, and homogenisers) used in the methods below must be made from **polypropylene plastic** (**PP** – confirm the polymer code 5 on the item) or **Teflon-coated stainless steel**. For the analysis of **CPCs**, all the equipment must be made from stainless steel.

However, it is recommended to use **PP** plastic tubes, buckets, and bags for ***both*** analysis types. PP tubes are safe to use as containers for samples being tested for CPC (e.g., bisphenols and BP-UV filters) and to use in the freeze-dryer under vacuum (which is how the freeze-drying process works). PP bags and buckets can also be used for sample collection and storage for both analysis types. PP plastic is often used for analyses like the abovementioned [41] because it does not interact with the samples. Some other plastic types may absorb the pollutants that are being tested for from the sample, leading to inaccurate results.

Each item (i.e., **all** the PP bags you will need, the total number of PP tubes for the samples you will be collecting, and all equipment such as tweezers, knives, spatulas, etc.) needs to be **washed with acid**, either HNO3 or HCl (supra pure 2-3 M) and **rinsed with ultra-pure water** (alternatively *deionised* water) five times, alternating between acid and then water, to avoid trace contamination. Separate the samples for each type of analysis, i.e., analysis for PHTEs and for CPCs, during sample collection. It is essential not to use stainless steel equipment for samples to be analysed for PHTEs, as the steel could potentially leave trace contamination on the samples. Similarly, do not use Teflon-coated or other plastic equipment on samples to be analysed for CPCs.

Nitrile, non-powdered gloves and 100% cotton laboratory coats must **always** be worn when handling the samples and equipment to avoid skin, metal, microplastic, or microfibre trace contamination.

Table 3.1: Summary of equipment material type for the analysis of different pollutants.

Item	Material type	
	PHTEs	CPCs
Laboratory coat	100% cotton	100% cotton
Gloves	Nitrile, non-powdered	Nitrile, non-powdered
Equipment, e.g.,tweezers, forceps, spatulas, knives, homogenisers	PP plastic or Teflon-coated stainless steel	Stainless steel, *alternatively* PP plastic
Tubes, bags, and buckets	PP plastic	PP plastic
Surfaces	PP plastic	Syainless steel, *alternatively* PP plastic
Freeze-dryer (see 3.3.1.4 g)	PP tube with parafilm	PP tube with foil

3.3.1.2. LABORATORY PREPARATION FOR BIVALVE SAMPLES

It is essential to process the samples as quickly as possible after collection to avoid any biological activity that could alter the chemical composition of the samples. Therefore, some preparation work can be done before sampling to ensure a speedy process. For example, acid-wash all the equipment **five** times under a fume hood. A fume hood is used to safely extract the acid fumes away from the researcher. Avoid touching your eyes, face, or skin when working with acid, and keep clean water close by to rinse off any spills. Prepare the correct number of PP bags and tubes before collecting the samples (*see **section 3.3.1.3** for sample collection*) and some bags to store the tubes in once washed. Also, prepare a few extra tubes and bags in case a bag gets ripped or a tube is dropped. Briefly: at least two PP bags are needed per site, one PP tube per sample, and at least four tubes for blanks per site (more details below). Ensure the PP tubes are the correct size/volume (25 ml tubes should suffice) to hold the sample and fit into the freeze-dryer. Alternatively, confirm the size of the tube with the laboratory technician.

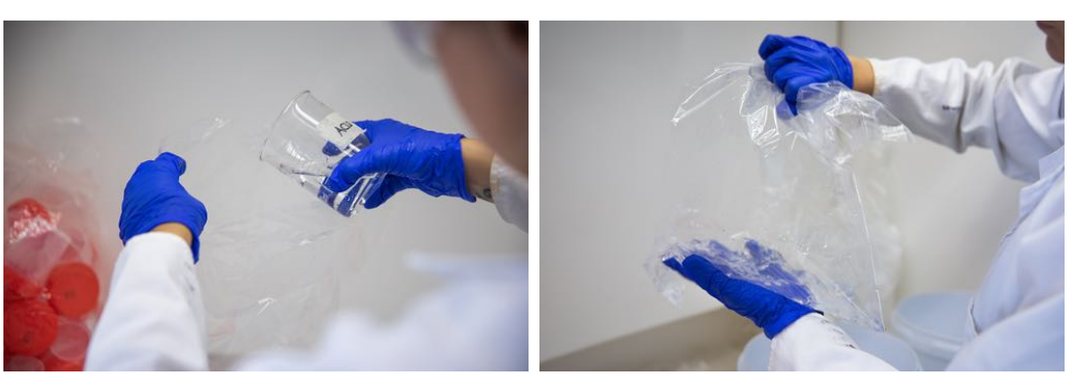
Table 3.2: Equipment list for laboratory preparation

YOU WILL NEED

- Ultra-pure water
- Two x 5 L PP buckets
- Acid: HNO3 or HCl (supra pure 2-3 M)
- A permanent marker
- A balance that displays at least three decimal places
- 25 ml PP tubes (one per sample and sample blanks)
- PP bags (50 x 35 cm or similar; two per site and some bags to put all the washed tubes in plus extra to have on hand)

HOW TO PREPARE LABORATORY EQUIPMENT (PRIOR TO FIELD COLLECTION):

a) *To prepare the bags*, work under a fume hood and wash the inside of each bag with acid, then rinse with ultra-pure water, and repeat five times. Use two 5 L PP buckets for the washing, one filled with ultra-pure water (about 3 – 5 cm deep to use the water sparingly) and one for acid (3 – 5 cm). A glass beaker can be used to pour acid into the bag. Tightly hold the opening of the bag closed with one hand and carefully shake the bag to ensure the acid reaches the entire inside of the bag. Do the same when rinsing with ultra-pure water. After repeating this five times, ensure you keep the bags closed (can be folded) and stored on a clean surface. Do not dry the bags but remove any excess water by shaking the bags upside down.



Acid-washing PP bags.

b) *To prepare the PP tubes*, work under a fume hood with one PP bucket filled with ultra-pure water (3 – 5 cm) and one filled with acid (3 – 5 cm). A few PP tubes can be washed at the same time. Open and place the tubes and lids in the bucket with acid. Wash the inside of each tube with the acid and then rinse each tube in ultra-pure water. After repeating this five times, shake the tube upside down to get rid of any excess water and close the lid. Store the closed PP tubes in a washed PP bag on a clean surface.

Acid-washing PP tubes.

c) After washing all the PP tubes, weigh and label (with the site number/name you have chosen and the sample number) all the PP tubes (*with their lids on)* and record the empty PP tube weights in a datasheet corresponding to the sample number. There are slight differences in the PP tubes' weights that can influence the results; therefore, it is important to keep a careful record of the tube numbers and their weights from this point forward. The weights are used to calculate the wet and dry weight of each sample at the end. Use a permanent marker to **clearly label the PP tube lids and the PP tubes, marking twice in different places on the tube.**

Weigh the empty PP tubes and record their weights.

d) Acid-wash (as above, wash in acid and rinse with ultra-pure water five times) all laboratory equipment (e.g., forceps, spatulas, knives, homogenisers) and leave them to dry under the laminar flow. Do not leave any equipment in the acid as this could harm the material or cause the stainless steel to rust. Clean all surfaces in preparation to receive samples from the field.

Clean equipment and workspace.

3.3.1.3. BIVALVE SAMPLE COLLECTION IN THE FIELD

Plan an exploratory visit to potential sampling sites before collecting the samples to assess the feasibility of a site for sampling, e.g., general safety, tidal cycle, and presence of your selected species. Remember to apply for a **sample permit** if needed. The number of sites is dependent on the available resources to cover the field and sample analyses costs.

Once the sampling sites are identified, plan the collection of samples accordingly, e.g., samples may need to be collected over a few consecutive days to account for the tide (i.e. it may be dangerous to sample during high tide). It is recommended to collect **blank field samples** at each site (*see* **Step f** *below for details*). This is to account for any background contamination due to handling the samples, e.g., potential trace contamination from the researcher or the environment, that may cause variation in the results. It is important to keep contamination in mind and work as cleanly and carefully as possible when handling the samples. Wear gloves if possible, but if they tear, e.g., on the rocks from where bivalves are collected, the samples can be collected with bare hands.

To minimise the risk of sample contamination, it is **advised** to process the samples immediately after collection, i.e., fresh. Therefore, allow for enough time after collection for sample preparation. If the samples cannot be prepared immediately after sample collection, ensure the samples are labelled clearly and frozen at -20°C or below as soon as they are brought back from the field. Also, ensure no sample contamination occurs while the samples are in the freezer and seal the PP bags with tape (e.g., clear duct tape). The samples must freeze completely solid to avoid any biological activity (e.g., rotting) that could alter the composition of the sample.

3.3.1.3. BIVALVE SAMPLE COLLECTION IN THE FIELD

Table 3.3: Equipment list for sample collection.

YOU WILL NEED

- An identification guide for identifying the study species
- Stickers (white/coloured)
- Washed PP bags
- Clean cooler box/es
- Aluminium foil
- Blank samples (collected in the field)
- Teflon-coated OR stainless steel utensils (e.g., knives)
- Washed 25 L PP bucket(s) (big enough to hold samples)
- A permanent marker
- Nitrile gloves
- Ruler or measuring tool
- Datasheet
- Pre-frozen ice packs (avoid ice cubes)

HOW TO COLLECT BIVALVES IN THE FIELD:

a) When collecting samples (minimum of n = 10 required per site or confirm with the laboratory), record the species, sampling location (GPS), weather conditions, pollution sources (e.g., in a bay near a river mouth or near a wastewater outlet), and any notes (e.g., in the case of mariculture samples, what they were grown in) on the site datasheet (*see Datasheet 1*).

b) If possible, apply random sampling within a site to avoid collecting all the samples from the same cluster.

c) Use a ruler or measuring tool to standardise the size of the samples to be collected, as large size differences may result in high data variability.

d) Use the Teflon-coated (for PHTEs analysis) **OR** stainless steel (for CPCs analysis) utensils, if necessary, to remove the animals from the rocks or structures, and rinse the samples in nearby clean rock pools to get rid of any sediment or organic matter. Place the samples in a labelled PP bag (with the date and site details) with separate bags per sample analysis type. Place the bags inside the 25 L bucket.

Collecting and removing samples from the rocks.

Collected samples inside a PP bag placed inside a 25 L bucket.

e) Once all the samples from the site are collected, take the samples to the cooler box and place the PP bags between ice packs. Ensure that the PP bags are sealed and labelled. Water or moisture may remove the permanent marker on the bags; therefore, label stickers are recommended. For easier identification between sites, coloured stickers can be used with a specific colour assigned to each site. Do not use a pencil to label PHTEs samples.

Collected samples placed in a cooler box with ice packs

f) Collect **blank field samples** while collecting the samples. Keep an open empty PP tube in the field for the time it takes to collect a sample. Close the tube and label it clearly as a field blank with the site name/number. Two blanks per site should suffice.

g) Transport the samples in the cooler box to the laboratory as soon as possible, ensuring they remain cold. Avoid using ice blocks as melted ice can contaminate samples.

3.3.1.4. BIVALVE SAMPLE PREPARATION IN THE LABORATORY FOR THE ANALYSIS OF POTENTIALLY HARMFUL TRACE ELEMENTS (PHTES) AND COMMON PLASTIC CONSTITUENTS (CPCS)

It is recommended to do all laboratory sample preparation under a laminar flow cabinet to avoid sample contamination, as microplastic particles and fibres, and potentially metal and rust particles, may be present in the air and dust. A laminar flow cabinet reduces the risk of sample contamination by smoothly blowing clean, filtered air over the samples. Alternatively, do all the sample preparation inside washed PP bags. It is also recommended to collect **blank laboratory samples** to account for any contamination that may occur while processing the samples in the laboratory.

Keep a glass beaker with ultra-pure water and a glass beaker with acid nearby in the laminar flow cabinet to clean equipment between samples. If samples are collected from different sites, make sure to clean the surfaces before preparing samples from a new site and regularly replace the ultra-pure water and acid. It is important to keep the samples cold during preparation to avoid any decaying and potential biological activity.

For the analysis of **PHTEs**, all the preparation work must be done on **PP plastic surfaces**, not on steel or metal surfaces, and do not let the samples come into contact with any type of metal. For the analysis of **CPCs**, all the preparation work must be done on **stainless steel surfaces**, or alternatively, a PP plastic surface. **Steps a – f** should be completed on the same day as sampling. If this is not possible, place the samples in a freezer as soon as possible after field collection and process them as soon as possible thereafter.

Note that the samples may be more difficult to open (or shuck) once frozen closed.

Table 3.4: Equipment list for sample processing. ***IMPORTANT:*** *choose the correct material type for handling the samples per analysis.*

YOU WILL NEED

- Polypropylene (PP) plastic OR stainless steel working surfaces (e.g., a PP plastic cutting board)
- Washed 25 ml PP tubes
- Acid: HNO3 **or** HCl (supra pure 2-3 M)
- Ultra-pure water
- Nitrile gloves
- Teflon-coated **OR** stainless steel forceps spatula and knives
- Glass beakers
- Teflon-coated **OR** stainless steel homogeniser
- Two x 5 L PP buckets
- Parafilm (similar to Glad wrap) **OR** foil cut into small squares to fit over the PP tubes
- Pre-frozen ice packs (avoid ice cubes)
- Laboratory sample blanks
- A balance that displays at least three decimal places

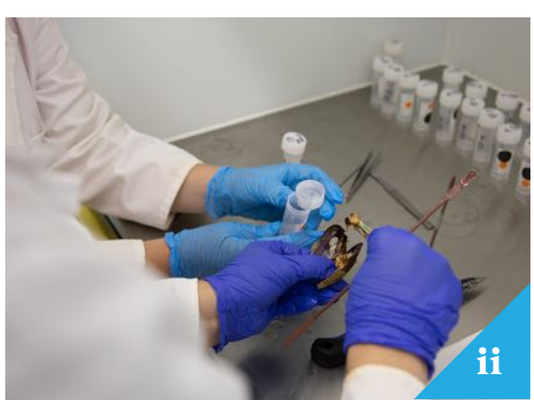
WHAT TO DO WITH SAMPLES AFTER FIELD COLLECTION:

a) Once back in the laboratory from the field, add frozen ice packs to the samples in the cooler box and keep it closed. This ensures the samples are kept cold while samples from one site are being processed if samples from multiple sites were collected. The cold will also help to open the samples as their shells should split from one another to make a small gap between them as they get cold. Use a PP plastic working surface for samples being prepared for PHTEs analysis ***OR*** a stainless steel working surface for samples being prepared for CPCs analysis.

b) Work under the laminar flow and place the PP bag with the samples to be processed from one site into a washed 5 L PP bucket with ice packs to keep them cold while processing the samples.

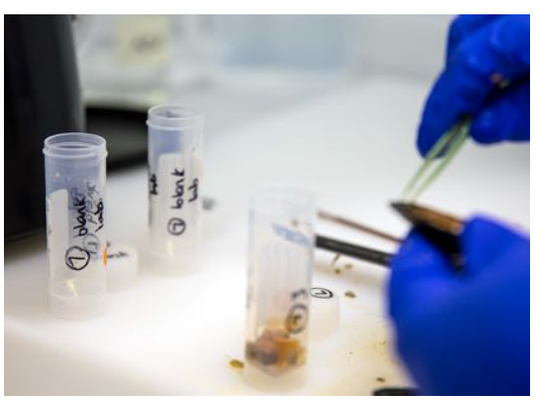
Samples ready to be processed placed in a 5 L bucket with frozen ice packs.

c) Two people most effectively accomplish sample processing. One person opens the sample using the Teflon-coated (for PHTEs analysis) **OR** stainless steel (for CPCs analysis) utensils, and another person scoops the sample (i.e., the entire organism, e.g., mussel or oyster) out of its shell into the labelled PP tube. **Close the lid** and place the tube in another PP bucket with ice packs to keep the samples, now inside the PP tubes, cold. Process all the samples from one site before starting to prepare samples from another site. To avoid cross-contamination, all the equipment, e.g., spatulas or knives, must be acid-washed and rinsed with ultra-pure water after each sample is prepared.



Opening a sample (i), scooping the sample out to place in a PP tube (ii), place the samples with closed lids in a 5 L bucket with an ice pack (iii) and acid-wash all equipment between samples (iv).

d) Collect **blank laboratory samples** while working under the laminar flow. Keep an open empty PP tube in the laminar flow cabinet for the time it takes to scoop out a sample. Close the tube and label it clearly as a **laboratory blank**. Two blank samples per site should suffice.

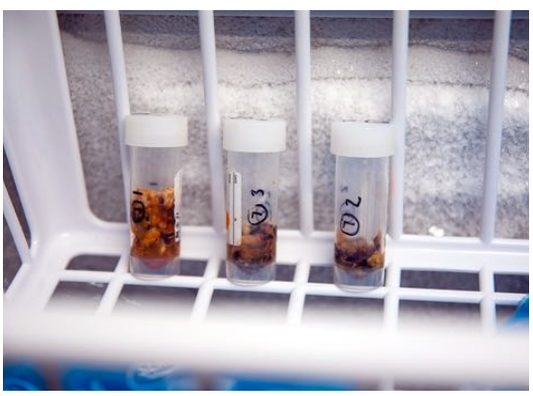


Lab blanks collected while processing a sample.

e) Once all the samples are scooped into PP tubes, weigh the samples and enter the weights on the datasheet with the corresponding weights of the empty tubes (*see Appendix 1 for a datasheet example*). Keep the samples cool by keeping them in the PP bucket with ice packs while weighing. Subtract the weight of the empty PP tubes from the total weight to obtain the wet sample weight (*always keep the lid on*):(PP tube & wet sample) – empty PP tube weight = wet sample weight

Weigh the samples and record the wet weight

f) Place the samples in a freezer of at least -20°C for a minimum of 12 hours (or overnight). Keep in mind that salt (e.g., in marine samples) lowers the freezing point of the samples, allowing them to thaw more easily. **Important:** The samples need to be **frozen solid in** the PP tubes before being placed in the freeze-dryer as liquid can damage the vacuum pump of the freeze-dryer. If there is any indication that the samples did not freeze properly after 24 hours, e.g., liquid is still visible, there is a possibility that the samples may be contaminated due to possible biological activity, in which case they need to be discarded.



Place the samples in the freezer.

g) Once frozen solid, remove the samples from the freezer and quickly replace the lids with **parafilm**, which is similar to Glad wrap, (for PHTEs analysis) **OR aluminium foil** (for CPCs analysis) under the laminar flow cabinet. Poke the **parafilm OR foil** with four to five holes of 1 – 2 mm to allow the moisture to escape in the freeze-dryer for the sample to dry effectively. Be careful not to let the samples thaw. If some of the samples froze against the lid, carefully scrape the samples off and move them down into the tube with a Teflon-coated (for PHTEs analysis) **OR** stainless steel (for CPCs analysis) spatula. Place the **labelled** lids in a PP bucket. The samples need to be placed in the freeze-dryer as quickly as possible to avoid thawing. Therefore, only after **Step h** below, acid-wash the lids in the PP buckets and place them in a closed, acid-washed PP bag to have them ready when the samples are dry. Take care not to remove the sample number on the lid, as each lid corresponds to a specific PP tube, which could influence the dry sample weight. Rewrite the sample numbers if necessary.

i

ii

*Scoop the sample down (i), use either aluminium foil (ii) OR parafilm (iii) to cover the PP tube and poke holes in them. Acid-wash the lids after **Step h** below (iv).*

h) Place the samples in the freeze-dryer at approximately 1 mbar and between -50 to -60°C for 24 to 48 hours, or until dry (*based on Christ model Beta 1 – 8 LSCBasic*). The settings will depend on the freeze-dryer model. It is advised to speak to a laboratory technician to ensure the correct settings are used.

Samples in the freeze-dryer.

i) The samples will appear dry and powdery and will pull away from the PP tube edges when ready. No moisture or bubbles should be visible. If there is still moisture visible in the tube, keep the samples in the freeze-dryer for longer. When the samples are completely dry, remove the samples from the freeze-dryer and replace the parafilm with the **corresponding** PP tube lid (*acid-washed and rinsed*) under the laminar flow cabinet.

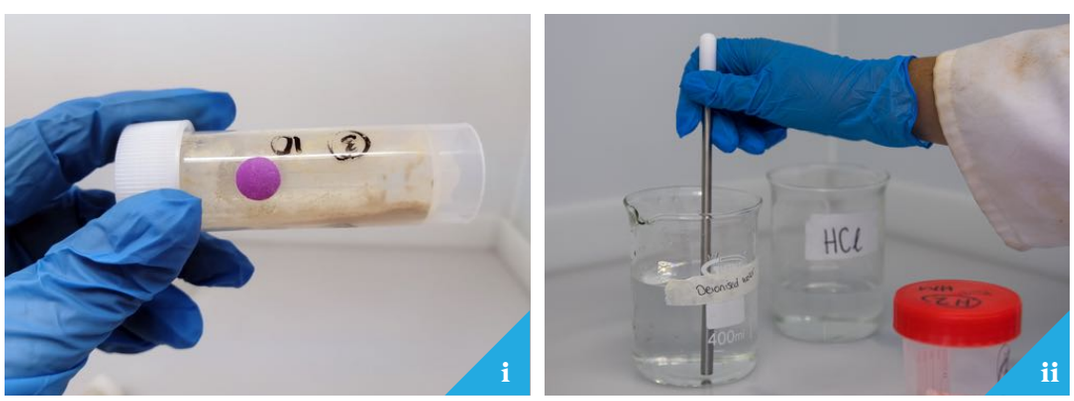

Dried sample with corresponding lid back on.

j) Once in this form, the samples can be stored at room temperature.

k) Weigh the samples and subtract the weight of the empty PP tubes to obtain the dry sample weight: (PP tube & dry sample) – empty PP tube weight = dry sample weight.

Weigh the samples and record the dry weight

l) Homogenise the samples in their PP tubes under the laminar flow cabinet using a **Teflon-coated pestle/ homogeniser** (PHTEs analysis) **OR** a **stainless steel pestle/ homogeniser** (CPCs analysis) until the samples are mixed well and appear as a fine powder. The homogeniser must be acid-washed and rinsed with ultra-pure water after each sample to avoid cross-contamination. We recommend homogenising the samples once dry as it is easier to crush the dry matter. There is also the benefit of doing it within the PP tube, which reduces movement of the sample and contamination.



Homogenised sample (i) and acid-washing equipment (ii).

m) Confirm the amount of each sample needed by the laboratory that will analyse the samples (*see 3.4. Sample Analyses*). Transfer the specified amount of sample as indicated by the laboratory into suitable containers, e.g., Eppendorf tubes, using a **Teflon-coated/PP plastic spatula** (PHTEs analysis) **OR stainless steel spatula** (CPCs analysis) and acid-wash and rinse after each sample. Most laboratories will require about 2 g per sample.

Some sample material transferred into Eppendorf tubes.

3.3.2. FISH

3.3.2.1. OVERVIEW AND MATERIALS

Below is a detailed, step-by-step description of methods for the preparation of samples, from sample collection to the freeze-drying process, specifically for fish to be analysed for PHTEs and/or CPCs using mass spectrometry (*section 3.4*.). A single sample consists of a piece of fish fillet, which is the part consumed by humans. It is **important** that all the necessary **permits and ethics approvals** have been obtained to continue with sample collection.

Take careful note of the type of material the equipment is made from for the different analysis types.**Table 3.5** summarises the equipment material for the analysis of each pollutant type. Keep in mind that the equipment material type in the photos used is for illustrative purposes ***only***; please confirm the material type you need to use based on the table below. For the analysis of **PHTEs**, all the equipment (knives, forceps, spatulas, and homogenisers) used in the methods below must be made from **polypropylene** plastic (PP – confirm the polymer code 5 on the item) or **Teflon-coated stainless steel**. For the analysis of CPCs, all the equipment must be made from **stainless steel**.

However, it is recommended to use **PP** plastic tubes, buckets, and bags for both analysis types. **PP** tubes are safe to use as containers for samples being tested for CPCs (e.g., bisphenols and BP-UV filters) and to use in the freeze-dryer under vacuum (which is how the freeze-drying process works). PP bags and buckets can also be used for sample collection and storage for both analysis types. PP plastic is often used for analyses like the abovementioned [41] and does not interact with the samples. Some other plastic types may absorb the pollutants that are being tested for from the samples, leading to inaccurate results.

Each item (i.e., **all** the PP bags you will need, the total number of PP tubes for the samples you will be collecting, and all equipment such as tweezers, knives, spatulas etc.) needs to be washed with acid, either HNO3 or HCl (supra pure 2-3 M), and rinsed with ultra-pure water (alternatively *deionised* water) five times, alternating between acid and then water, to avoid trace contamination. Separate the samples for each type of analysis, i.e., analysis for PHTEs and for CPCs, during sample collection. It is essential **not** to use stainless steel equipment for samples to be analysed for PHTEs, as the steel could potentially leave trace contamination on the samples. Similarly, do not use Teflon-coated or other plastic equipment on samples to be analysed for CPCs. Nitrile, non-powdered gloves and 100% cotton laboratory coats must **always** be worn when handling the samples and equipment to avoid skin, metal, microplastic, or microfibre trace contamination.

Table 3.5: Summary of equipment material type for the analysis of different pollutants.

Item	Material type	
	PHTEs	CPCs
Laboratory coat	100% cotton	100% cotton
Gloves	Nitrile, non-powdered	Nitrile, non-powdered
Equipment, e.g.,tweezers, forceps, spatulas, knives, homogenisers	PP plastic or Teflon-coated stainless steel	Stainless steel, *alternatively* PP plastic
Tubes, bags, and buckets	PP plastic	PP plastic
Surfaces	PP plastic	Stainless steel, *alternatively* PP plastic
Freeze-dryer (see 3.3.2.4 h)	PP tube with parafilm	PP tube with foil

3.3.2.2. LABORATORY PREPARATION FOR FISH SAMPLES

It is essential to process the samples as quickly as possible after collection to avoid any biological activity that could alter the chemical composition of the samples. Therefore, some preparation work can be done beforehand to ensure a speedy process. For example, acid-wash all the equipment **five** times under a fume hood. A fume hood is used to safely extract the acid fumes away from the researcher. Avoid touching your eyes, face, or skin when working with acid, and keep clean water close by to rinse off any spills. Prepare the correct number of PP tubes before collecting the samples (see **section 3.3.2.3** for sample collection) and bags to store the tubes once washed. Also, prepare a few extra tubes and bags in case a bag gets ripped or a tube is dropped. Briefly: 1 PP tube per sample is needed, at least 2 tubes for blanks per site (more details below) and 5 to 10 bags. Ensure the PP tubes are the correct size/volume (180 ml tubes should suffice) to hold the sample and fit into the freeze-dryer. Alternatively, confirm the size of the tube with the laboratory technician.

Table 3.6: Equipment list for laboratory preparation.

YOU WILL NEED

- Ultra-pure water
- Acid: HNO_3 or HCl (supra pure 2-3 M)
- A balance that displays at least three decimal places
- PP bags (50 x 35 cm or similar; 5 – 10 bags)
- Two x 5 L PP buckets
- A permanent marker
- 180 ml PP tubes (one per sample and sample blanks)

HOW TO PREPARE LABORATORY EQUIPMENT (PRIOR TO FIELD COLLECTION)

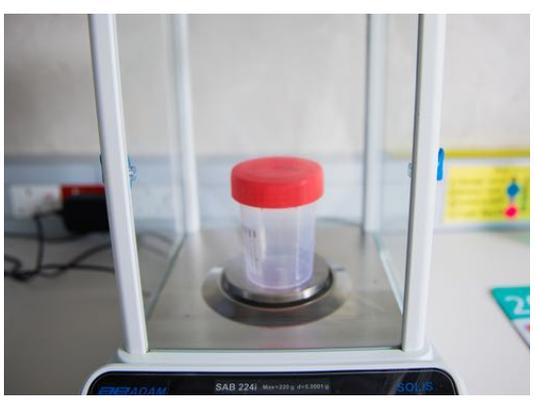
a) *To prepare the bags*, work under a fume hood and wash the inside of each bag with acid, then rinse with ultra-pure water, and repeat five times. Use two 5 L PP buckets for the washing, one filled with ultra-pure water (about 3 – 5 cm deep to use the water sparingly) and one for acid (3 – 5 cm). A glass beaker can be used to pour acid into the bag. Tightly hold the opening of the bag closed with one hand and carefully shake the bag to ensure the acid reaches the entire inside of the bag. Do the same when rinsing with ultra-pure water. After repeating this five times, ensure you keep the bags closed (can be folded) and stored on a clean surface. Do not dry the bags but remove any excess water by shaking the bags upside down.

Acid-washing PP bags.

b) *To prepare the PP tubes*, work under a fume hood with one PP bucket filled with ultra-pure water (3 – 5 cm) and one filled with acid (3 – 5 cm). A few PP tubes can be washed at the same time. Open and place the tubes and lids in the bucket with acid. Wash the inside of each tube with the acid and then rinse each tube in ultra-pure water. After repeating this five times, shake the tube upside down to get rid of any excess water and close the lid. Store the closed PP tubes in a washed PP bag on a clean surface.

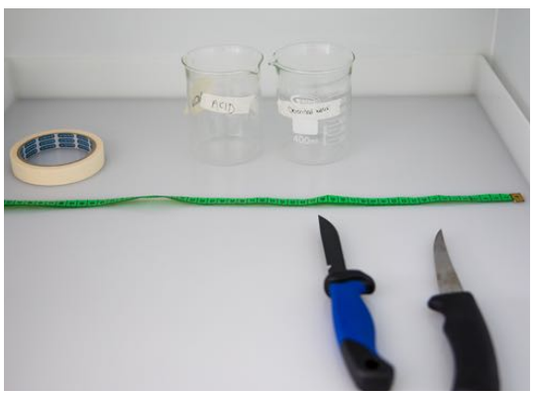
Acid-washing PP tubes.

c) After washing all the PP tubes, weigh and label (with the site number/name you have chosen and the sample number) all the PP tubes *(with their lids on)* and record the empty PP tube weights in a datasheet corresponding to the sample number. There are slight differences in the PP tubes' weights that can influence the results; therefore, it is important to keep a careful record of the tube numbers and their weights from this point forward. The weights are used to calculate the wet and dry weight of each sample at the end. Use a permanent marker to **clearly label the PP tube lids and the PP tubes, marking twice in different places on the tube.**



Weigh and record the weights of the empty PP tubes.

d) Acid-wash (as above, wash in acid and rinse with ultra-pure water five times) all laboratory equipment (e.g., forceps, spatulas, knives, homogenisers) and leave to dry under the laminar flow. Do not leave any equipment in the acid as this could harm the material or cause the stainless steel to rust. Clean all surfaces in preparation to receive samples from the field.



Clean equipment and workspace.

3.3.2.3. FISH SAMPLE COLLECTION

There are various ways to obtain fish samples, but always ensure the fish is fresh and is kept cold. Fish can be bought from local markets or a collaboration can be established with local anglers to catch the desired species. Alternatively, the facility or university undertaking the study can catch or collect the samples if they have the means to do so (e.g., fishing equipment, a boat). **IMPORTANT:** ensure the correct species identification is made of the fish and that the catch location is known and recorded.

To minimise the risk of sample contamination, it is **advised** to process the samples immediately after being sampled, i.e., fresh. Therefore, allow for enough time after collection for sample preparation. If the samples cannot be prepared immediately after sample collection, ensure the samples are labelled clearly and frozen at -20 °C or below as soon as they are brought back from the field. The samples must freeze completely solid to avoid any biological activity that could alter the composition of the sample (e.g., rotting).

Table 3.7: Equipment list for sample collection.

YOU WILL NEED

- An identification guide for identifying the study species
- Washed 25 L PP bucket(s) (big enough to hold samples) or cooler box/es
- Clean cooler box/es
- Nitrile gloves
- Datasheet
- Pre-frozen ice packs (avoid ice cubes)

WHAT TO DO WHEN COLLECTING OR RECEIVING FISH SAMPLES:

a) When catching or collecting samples (minimum of n = 10 required per fish species or confirm with the laboratory), record the species, sampling location (GPS), weather conditions, pollution sources (e.g., in a bay near a river mouth or near a wastewater outlet), and any other notes on the site datasheet (see *Datasheet 1*). If samples were bought or donated, note down the date and ask for any additional information, e.g., GPS locations of where the fish was caught, how the fish was caught, and confirm the species.

b) Place the samples in the 25 L bucket (if the species is small enough) or place the samples in the cooler box. Ensure the fish are kept cool by adding ice packs to the container and transport the samples to the laboratory.

3.3.2.4. FISH SAMPLE PREPARATION FOR THE ANALYSIS OF POTENTIALLY HARMFUL TRACE ELEMENTS (PHTES) AND COMMON PLASTIC CONSTITUENTS (CPCS)

It is recommended to do all laboratory sample preparation under a laminar flow cabinet to avoid sample contamination, as microplastic particles and fibres, and potentially metal and rust particles, may be present in the air and dust.

A laminar flow cabinet reduces the risk of sample contamination by smoothly blowing clean, filtered air over the samples. Alternatively, do all the sample preparation inside washed PP bags. It is also recommended to collect **blank laboratory samples** to account for any contamination that may occur while processing the samples in the laboratory.

Keep a glass beaker with ultra-pure water and a glass beaker with acid nearby in the laminar flow cabinet to clean equipment between samples. Ensure that all surfaces are cleaned after **each sample** and regularly replace the ultra-pure water and acid. It is important to keep the samples cold during preparation to avoid any decaying and potential biological activity that could alter the chemical composition of the samples.

For the analysis of **PHTEs**, all the preparation work must be done on PP plastic surfaces, not on steel or metal surfaces, and do not let the samples come into contact with any type of metal. For the analysis of **CPCs**, all the preparation work must be done on **stainless steel surfaces**, or alternatively, a PP plastic surface.

Do not work on other plastic surfaces or let the samples come into contact with any plastic other than PP plastic (*see explanation in 3.3.1.1*). **Steps a – g** should be completed on the same day as sampling or on the day they were received. If the samples were received frozen, carefully thaw the samples just enough to work with them. Do not let them completely thaw out. If it is not possible to process the samples on the same day, e.g., due to sampling operations, place the samples in a freezer and process them as soon as possible.

Table 3.8: Equipment list for sample processing. ***IMPORTANT:*** *choose the correct material type for handling the samples per analysis.*

YOU WILL NEED

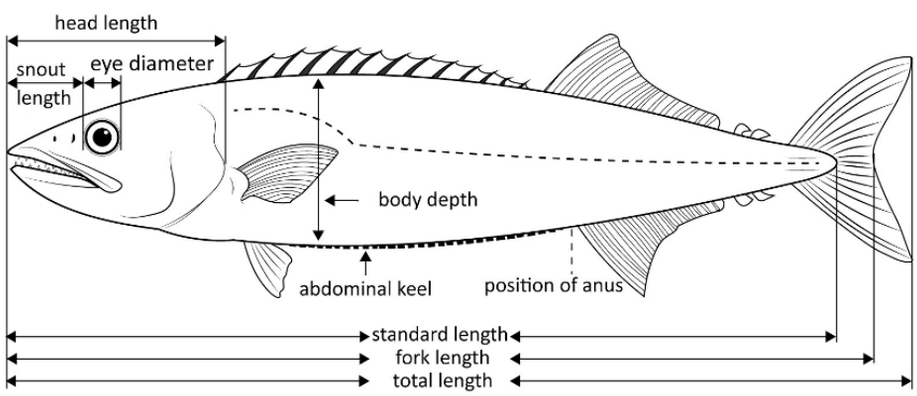
- Polypropylene (PP) **OR** stainless steel working surfaces (e.g., a PP plastic cutting board)
- Teflon-coated **OR** stainless steel forceps spatula and knives
- Acid: HNO3 or HCl (supra pure 2-3 M)
- Teflon-coated **OR** stainless steel homogeniser
- Nitrile gloves
- Washed 180 ml PP tubes
- Glass beakers
- Ultra-pure water
- Two x 5 L PP buckets
- Parafilm (similar to Glad wrap) **OR** foil cut into small squares to fit over the PP tubes
- Pre-frozen ice packs (avoid ice cubes)
- Thin rope
- Masking (or painter's) tape
- Permanent marker
- Measuring tape
- Laboratory sample blanks
- Scale and a balance that displays at least three decimal places

WHAT TO DO WITH SAMPLES AFTER FIELD COLLECTION:

a) Once in the laboratory, add frozen ice packs to the samples in the cooler box and keep it closed. This ensures the samples are kept cold while being processed. Use a PP plastic working surface for samples being prepared for PHTEs analysis **OR** a stainless steel working surface for samples being prepared for CPCs analysis.

b) Work under the laminar flow and record biometric data on a datasheet for each fish before cutting a sample, i.e., piece of fillet. Zero or tare a washed 5 L PP bucket on a scale and then place the fish into the bucket and record the weight. Measure the length of the fish with a measuring tape as indicated in the illustration below. It is advised to measure at least the fork **and** total length. You can label or mark the fish here with the corresponding sample number or at **Step e**.

Weigh (i), measure (ii), and record the biometric data on a datasheet (iii) for each fish/sample.



Explanation of different fish measurements.

c) OPTIONAL: To record the sex of the fish, you will need to open the fish by making a shallow incision along its stomach. Insert the tip of the knife at the anus, cutting up along the abdominal keel to between the pelvic fins (i). Open the fish and identify the gonads (reproductive organs) located above the intestine and below the swim bladder, positioned at the top of the gut cavity (ii). Females will have orange/yellow ovaries with visible veins and arteries, and males will have white testes (*see Box 1*). **IMPORTANT:** use a Teflon-coated knife for samples to be analysed for PHTEs **OR** a stainless steel knife for samples to be analysed for CPCs.

BOX 1: DETERMINING THE SEX OF A FISH

Literature on the reproductive biology of the species that is being sampled should be consulted. There is general protocol for determining the sex and stage of the gonads, however, there is variation in how the gonads look depending on what stage they are in and there is variation among species. Thus, in most of the literature on fish reproduction, a table describing what to look for in the species which is in question is provided.

Colour of gonads: Males - white and strappy and can often see the sperm; females – yellow/ orange and can often see the oocytes (little eggs).

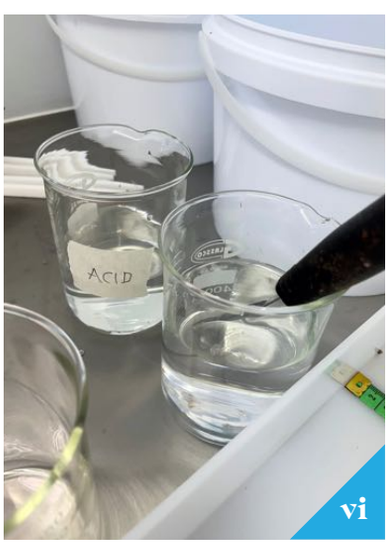
Make an incision along the stomach of the fish (i) and open the fish to identify the gonads at the top of the gut cavity (ii).

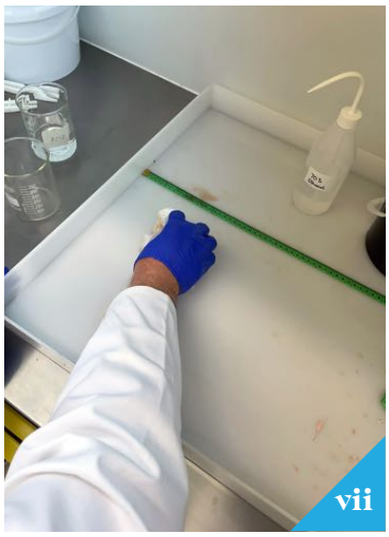
d) Collect **blank laboratory samples** while working under the laminar flow. Keep an open empty PP tube in the laminar flow cabinet for the time it takes to cut a sample as described below (*e*). Close the tube and label it clearly as a **laboratory blank**. Two blank samples should suffice.

Laboratory blank sample to be kept open while processing one sample.

e) To obtain a piece of fish fillet from which a sample will be cut, insert the point of the knife behind the head (next to the start of the dorsal spines) and run the knife along the backbone, cutting towards the tail (*i*). Cut through the bones enclosing the stomach cavity and remove the fillet from the bone. To remove the skin, place the piece of flesh skin down on the working surface (**PP or stainless steel**), place the knife at the tail section and gently cut through the meat to the skin (but not through the skin) and run the knife towards the head section (*ii*). Cut a piece of fillet about 5 cm long (*iii*) and place it into the 180 ml PP tube (*iv*). Mark or label each fish to the **corresponding** sample number and biometric data on a datasheet. Use a piece of thin rope and tie a knot around the tail of the fish. Write the sample number on a piece of masking tape and stick it to the rope (*v*). To avoid cross-contamination, all the equipment, e.g., knives, must be acid-washed and rinsed with ultra-pure water after each sample (*vi*). Also, wipe down the working area (*vii*).

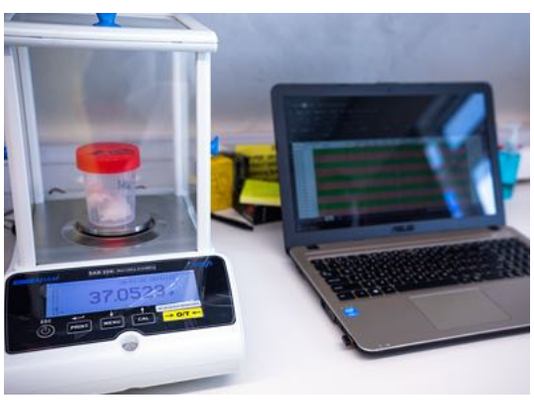
IMPORTANT: use a Teflon-coated knife for samples to be analysed for PHTE OR a stainless steel knife for samples to be analysed for CPCs.





Obtaining a piece of fish fillet for a sample (i – vii).

f) Weigh the tube with the sample and enter the weights on a data sheet (*this can be done directly into Excel, see Appendix 1 as an example*) with all the empty tube weights and corresponding sample numbers. Subtract the weight of the empty PP tubes to obtain the wet sample weight (*always keep the lids on*):
(PP tube & wet sample) – empty PP tube weight = wet sample weight.



Weigh and record the wet sample weight.

g) Place the samples in a freezer of at least -20°C for a minimum of 12 hours (or overnight). Keep in mind that salt (e.g., in marine samples) lowers the freezing point of the samples, allowing them to thaw more easily. **Important:** The samples need to be **frozen solid** in the PP tubes to be placed in the freeze-dryer as liquid can damage the vacuum pump of the freeze-dryer. If there is any indication that the samples did not freeze properly after 24 hours, e.g., liquid is still visible, there is a possibility that the samples may be contaminated due to possible biological activity, in which case they need to be discarded.

Place the samples in the freezer.

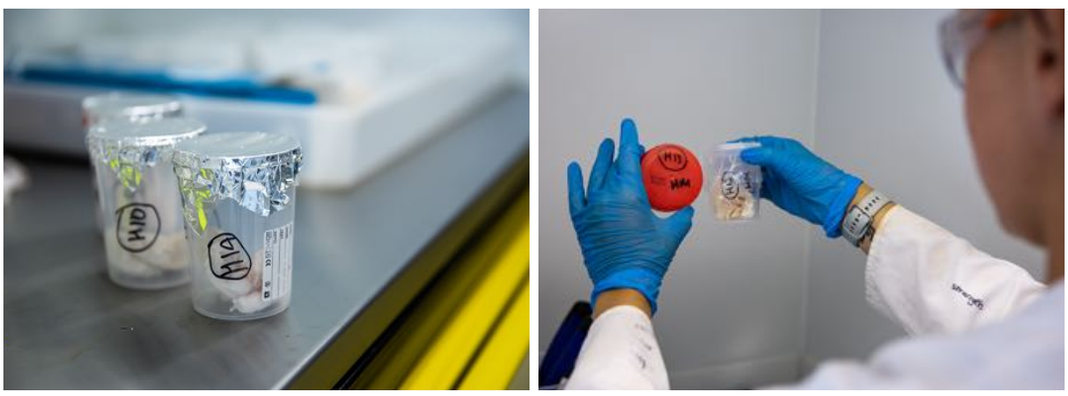
h) Once frozen solid, remove the samples from the freezer and quickly replace the lids with **parafilm**, which is similar to Glad wrap, (for PHTEs analysis) **OR aluminium foil** (for CPCs analysis) under the laminar flow cabinet. Poke the **parafilm OR foil** with four to five holes of 1 – 2 mm to allow the moisture to escape in the freeze-dryer for the sample to dry effectively. Be careful not to let the samples thaw. If some of the samples froze against the lid, carefully scrape the samples off and move them down with a Teflon-coated (for PHTEs analysis) **OR** stainless steel (CPCs analysis) spatula. Place the **labelled** lids in a PP bucket. The samples need to be placed in the freeze-dryer as quickly as possible to avoid thawing. Therefore, only after **Step *i*** below, acid-wash the lids in the PP buckets and place them in a closed, acid-washed PP bag to have them ready when the samples are dry. Take care not to remove the sample number on the lid, as each lid corresponds to a specific PP tube, which could influence the dry sample weight. Rewrite the sample numbers if necessary.

Use either parafilm (i) OR aluminium foil (ii) to cover the PP tubes and poke holes in them (iii and iv). Acid-wash the lids after Step i below (v).

i) Place the samples in the freeze-dryer at approximately 1 mbar and between-50 to -60 °C for 24 to 48 hours, or until dry (based on *Christ model Beta 1 – 8 LSCBasic*). The settings will depend on the freeze-dryer model, and it is advised to speak to a laboratory technician to ensure the correct settings are used.

Samples in the freeze-dryer.

j) The samples will appear dry and powdery and will pull away from the PP tube edges when ready. No moisture or bubbles should be visible. If there is still moisture visible, keep the samples in the freeze-dryer for longer. When the samples are completely dry, remove the samples from the freeze-dryer and replace the parafilm **OR** foil with the **corresponding** PP tube lid (*acid-washed and rinsed*) under the laminar flow cabinet.



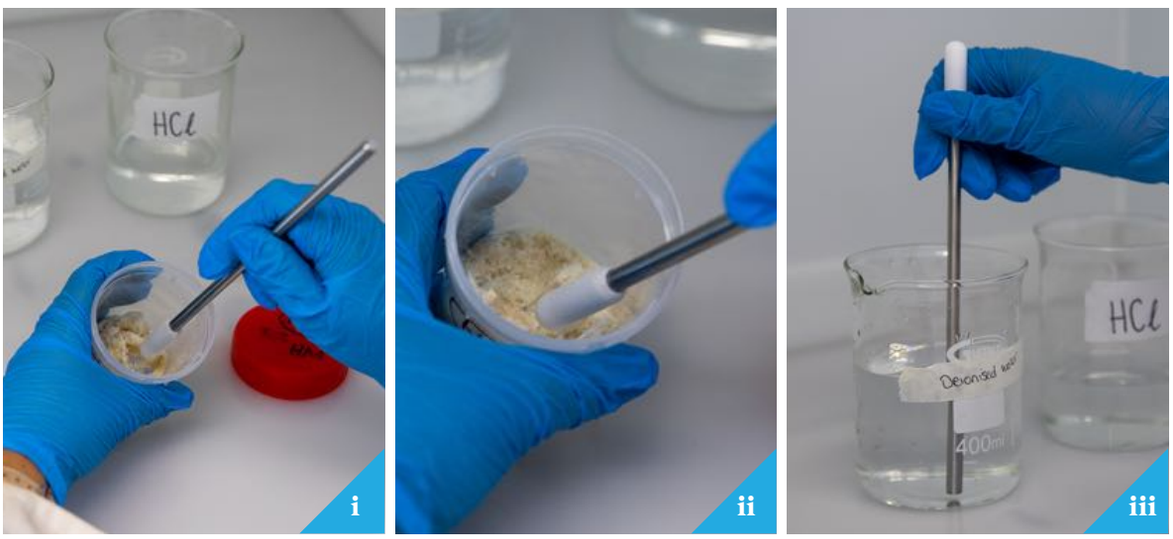
Remove the parafilm or foil and place the corresponding lids on each tube.

k) Once in this form, the samples can be stored at room temperature.

l) Weigh the samples and subtract the weight of the empty PP tubes to obtain the dry sample weight: (PP tube & dry sample) – empty PP tube weight = dry sample weight

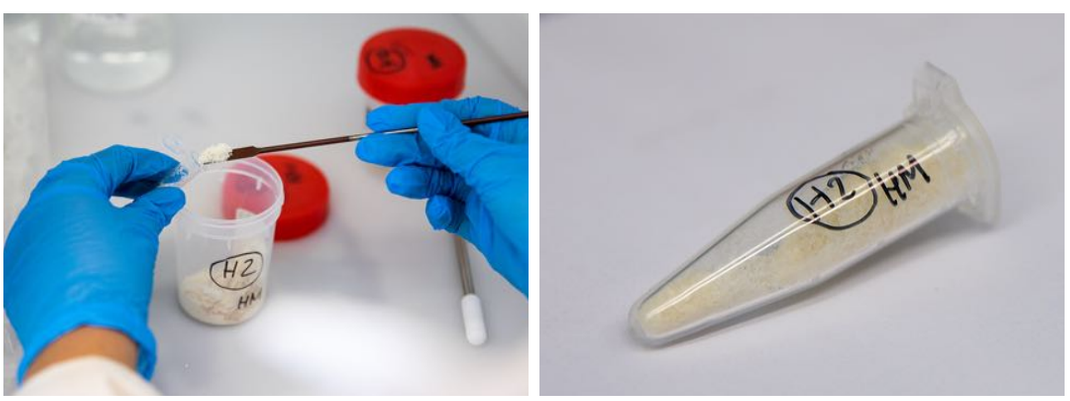
Weigh the samples and record the dry weight.

m) Homogenise the samples in their PP tubes under the laminar flow cabinet using a **Teflon-coated** pestle/ homogeniser (for PHTEs analysis) **OR stainless steel** pestle/homogeniser (for CPCs analysis) until the samples are mixed well and appear as a fine powder. The homogeniser must be acid-washed and rinsed with ultra-pure water after each sample to avoid cross-contamination. We recommend homogenising the samples once dry as it is easier to crush the dry matter. There is also the benefit of doing it within the PP tube, which reduces movement of the sample and contamination.



Homogenise the samples (i -ii) and acid-wash the equipment between samples (iii).

n) Confirm the amount of each sample needed by the laboratory that will analyse the samples (see **3.4. Sample analyses**). Transfer the amount of sample as indicated by the laboratory into suitable containers, e.g., Eppendorf tubes, using a **Teflon-coated/PP plastic** (for PHTEs analysis) **OR stainless steel** (for CPCs analysis) spatula and acid-wash and rinse after each sample. Most laboratories will require about 2 g per sample.



Transfer a small amount of sample into an Eppendorf tube.

3.4. SAMPLE ANALYSIS

There are now two choices for where the samples could be analysed: 1) the **freeze-dried** samples can be sent to an external laboratory to be analysed for PHTEs and CPCs; 2) if the facility or university undertaking this study has the expertise and equipment to carry out the analyses, the **freeze-dried** samples can be analysed in-house. As a suggestion, samples can be analysed for PHTEs using inductively coupled plasma mass-spectrometry (ICP-MS) and for CPCs using ultra-high performance liquid chromatography-mass spectrometry (LC-MS).

REFERENCES

1. GESAMP. 2019. Guidelines for the monitoring and assessment of plastic litter in the ocean. (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/ UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Reports & Studies. GESAMP No. **99**, 130 p.

2. Cole, M., Lindeque, P., Halsband, C. & Galloway, T. S. 2011. Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin* **62**, 2588–2597.

3. Van Sebille, E. et al. 2015. A global inventory of small floating plastic debris. *Environmental Research Letters* **10**, 124006.

4. GESAMP. 2016. Sources, fate and effects of microplastics in the marine environment: part two of a global assessment. (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/ UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Reports & Studies. GESAMP No. **93**, 220 p.

5. Gregory, M. R. 1978. Accumulation and distribution of virgin plastic granules on New Zealand beaches. *New Zealand Journal of Marine and Freshwater Research* **12**, 399–414.

6. Zitko, V. & Hanlon, M. 1991. Another Source of Pollution by Plastics: Skin Cleaners with Plastic Scrubbers Pesticide Residues in Sediments from the West Coast of India. *Marine Pollution Bulletin* **22**, 41–42.

7. Andrady, A. L. 2011. Microplastics in the marine environment. *Marine Pollution Bulletin* **62**, 1596–1605.

8. Browne, M. A., Galloway, T. & Thompson, R. 2007. Microplastic - an emerging contaminant of potential concern? *Integrated Environmental Assessment and Management* **3**, 559–566.

9. Duis, K. & Coors, A. 2016. Microplastics in the aquatic and terrestrial environment: sources (with a specific focus on personal care products), fate and effects. *Environmental Sciences Europe* **28**, 1–25.

10. Hidalgo-Ruz, V., Gutow, L., Thompson, R. C. & Thiel, M. 2012. Microplastics in the marine environment: A review of the methods used for identification and quantification. *Environmental Science and Technology* **46**, 3060–3075.

11. Barnes, D. K. A., Galgani, F., Thompson, R. C. & Barlaz, M. 2009. Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**, 1985–1998.

12. Andrady, A. L. 2015. Persistence of plastic litter in the oceans. in *Marine Anthropogenic Litter* (eds. Bergman, M., Gutow, L. & Klages, M.), Springer, 57–72.

13. Carbery, M., O'Connor, W. & Palanisami, T. 2018. Trophic transfer of microplastics and mixed contaminants in the marine food web and implications for human health. *Environment International* **115**, 400–409.

14. Rochman, C. M. et al. Classify plastic waste as hazardous. *Nature* **494**, 169–171.

15. Revel, M., Châtel, A. & Mouneyrac, C. 2018. Micro(nano)plastics: A threat to human health? *Current Opinion in Environmental Science & Health* **1**, 17–23.

16. OECD. 2004. Emission Scenario Document on Plastic Additives. Series on Emission Scenario Documents, No. 3. *OECD Environmental Health and Safety Publications*. Environment Directorate, Paris

17. Lithner, D., Larsson, A. & Dave, G. 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Science of the Total Environment* **409**, 3309–3324.

18. Rochman, C. M. 2015. The Complex Mixture, Fate and Toxicity of Chemicals Associated with Plastic Debris in the Marine Environment. In *Marine Anthropogenic Litter* (eds. Bergmann, M., Gutow, L., & Klages, M.), Springer, 117–140.

19. EU, 2002. European Union Risk Assessment Report. Styrene – Part I – Environment. **Volume 27**. Institute for Health and Consumer Protection. European Chemicals Bureau, Luxembourg: Office for Official Publications of the European Communities EUR 20541 EN.

20. Gennaro, V., Ceppi, M., Crosignani, P. & Montanaro, F. 2008. Reanalysis of updated mortality among vinyl and polyvinyl chloride workers: Confirmation of historical evidence and new findings. *BMC Public Health* **8**, 1–8.

21. Rios, L. M., Jones, P. R., Moore, C. & Narayan, U. V. 2010. Quantitation of persistent organic pollutants adsorbed on plastic debris from the Northern Pacific Gyre's 'eastern garbage patch'. *Journal of Environmental Monitoring* **12**, 2226–2236.

22. Savoca, M. S., McInturf, A. G. & Hazen, E. L. 2021. Plastic ingestion by marine fish is widespread and increasing. *Global Change Biology* **27**, 2188–2199.

23. Farrell, P. & Nelson, K. 2013. Trophic level transfer of microplastic: Mytilus edulis (L.) to Carcinus maenas (L.). *Environmental Pollution* **177**, 1–3.

24. Van Cauwenberghe, L. & Janssen, C. R. 2014. Microplastics in bivalves cultured for human consumption. *Environmental Pollution* **193**, 65–70.

25. Batel, A., Linti, F., Scherer, M., Erdinger, L. & Braunbeck, T. 2016. Transfer of benzo[a]pyrene from microplastics to *Artemia nauplii* and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants. *Environmental Toxicology and Chemistry* **35**, 1656–1666.

26. Gallo, F. et al. 2018. Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preventive measures. *Environmental Sciences Europe* **30**, 13.

27. Rochman, C. M. et al. 2015. Anthropogenic debris in seafood: Plastic debris and fibers from textiles in fish and bivalves sold for human consumption. *Scientific Reports* **5**, 14340.

28. Van Cauwenberghe, L., Claessens, M., 2015. Vandegehuchte, M. B. & Janssen, C. R. Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural habitats. *Environmental Pollution* **199**, 10–17.

29. Li, J. et al. 2019. Using mussel as a global bioindicator of coastal microplastic pollution. *Environmental Pollution* **244**, 522–533.

30. Boening, D. W. 1999. An evaluation of bivalves as biomonitors of heavy metals pollution in marine waters. *Environmental Monitoring and Assessment* **55**, 459–470.

31. Goldberg, E. D. & Bertine, K. K. 2000. Beyond the Mussel Watch - New directions for monitoring marine pollution. Science of the *Total Environment* **247**, 165–174.

32. Hédouin, L., Pringault, O., Bustamante, P., Fichez, R. & Warnau, M. 2011. Validation of two tropical marine bivalves as bioindicators of mining contamination in the New Caledonia lagoon: Field transplantation experiments. *Water Research* **45**, 483–496.

33. Góngora-Gómez, A. M. et al. 2017. Heavy-metal contents in oysters (*Crassostrea gigas*) cultivated on the southeastern coast of the Gulf of California, Mexico. *Hidrobiologica* **27**, 219–227.

34. Shaari, H., Raven, B., Sultan, K., Mohammad, Y. & Yunus, K. 2016. Status of heavy metals concentrations in oysters (*Crassostrea sp.*) from Setiu Wetlands, Terengganu, Malaysia. *Sains Malaysiana* **45**, 417–424.

35. Mackay, N. J. et al. 1975. Heavy Metals in Cultivated Oysters from the Estuaries of New South Wales. *Austrailan Journal Of Marine and Freshwater Research* **26**, 31–46.

36. Ochoa, V., Barata, C. & Riva, M. C. 2013. Heavy metal content in oysters (*Crassostrea gigas*) cultured in the Ebro Delta in Catalonia, Spain. *Environmental Monitoring and Assessment* **185**, 6783–6792.

37. Beyer, J. et al. 2017. Blue mussels (*Mytilus edulis spp.*) as sentinel organisms in coastal pollution monitoring: A review. *Marine Environmental Research* **130**, 338–365.

38. Castro, G. et al. 2021. Occurrence of bisphenols and benzophenone UV filters in wild brown mussels (*Perna perna*) from Algoa Bay in South Africa. *Science of The Total Environment* **813**, 152571.

39. Storelli, M. M. 2008. Potential human health risks from metals (Hg, Cd, and Pb) and polychlorinated biphenyls (PCBs) via seafood consumption: Estimation of target hazard quotients (THQs) and toxic equivalents (TEQs). *Food and Chemical Toxicology* **46**, 2782–2788.

40. Repossi, A., Farabegoli, F., Gazzotti, T., Zironi, E. & Pagliuca, G. 2016. Bisphenol A in edible part of seafood. *Italian Journal of Food Safety* **5**, 2.

41. González-Rubio, S. *et al*. 2020. Bioaccumulation potential of bisphenols and benzophenone UV filters: A multiresidue approach in raptor tissues. *Science of the Total Environment* **741**, 140330.

DATASHEET 1: BIOLOGICAL SAMPLE COLLECTION

Data collector details

Name: _____ Date: _____

Organisation/Institution: _____

**ensure to only use clean, pre-washed equipment*

Sample information

Invertebrates ☐ Fish ☐ Other ☐

Species: _____ Sampling location: _____

_____ GPS coordinates: _____

Sampling conditions & observations (as described in manual): _____

Number of samples collected: _____

Labelled from: _____ To: _____

Tissue type collected (whole/fillet - indicate on sample label): ☐

Number of samples for CPCs: ☐

Number of samples for PHTEs: ☐

Collaborators on research (specify researcher and organisation):

_____

_____

_____

Other:

_____

_____

_____

APPENDIX 1: EXAMPLE OF DATA RECORDS

Table A1: Example of fish data that can be recorded while processing the samples

Date	Location	Sample ID*	Species	Sex	Fish weight/ mass (g)	Total length (cm)	Fork length (cm)	Empty container (g)	Wet weight with container (g)	Sample wet weight (g)	Dry weight with container (g)	Sample dry weight (g)
2022/02/02	Algoa Bay	C1 PHTE	*Argyrozona argyrozona*	Male	315.76	30.5	26.9	21.0306	43.8115	22.7809	25.8562	4.8256
2022/02/03	Algoa Bay	C2 PHTE	*Argyrozona argyrozona*	Female	492.68	34.7	32	21.6191	43.9114	22.2923	28.2001	6.581
2022/02/03	Algoa Bay	C2 CPC	*Argyrozona argyrozona*	Male	315.76	33.7	29	21.5236	44.0258	22.5022	27.8956	6.3717
Etc.												

*label samples for different analysis types

NOTES:

NOTES:

SAMPLE PREPARATION MANUAL

FOR THE ANALYSIS OF PLASTIC-RELATED POLLUTANTS

EDITED BY
AMAREIN J FOURIE AND DANICA MARLIN

2022 EDITION

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