Sampling guidelines to assess plastic ingestion in ACAP species

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SUMMARY

These guidelines provide a standardized approach for sampling ACAP species to assess plastic ingestion (macro and microplastics, as well as chemical compounds) with an array of sample type choices that should enable collection in diverse settings. Samples can be collected from dead beached or by-caught specimens, live and dead animals in breeding sites or rehabilitation centres, as well as non-invasively by sampling fresh scats from nests, regurgitated boluses or unviable or hatched eggs. Given the particular susceptibility of ACAP species to plastic ingestion and the increasing prevalence of this problem worldwide, collecting samples to assess plastic ingestion should be considered whenever an opportunity presents. *Using standardized protocols increases the consistency and representativeness of results and allows comparisons between species and detection of large-scale spatiotemporal patterns*. Target research and surveillance options include:

1. Macroplastics (>5mm): can be assessed from stomach contents in dead birds, regurgitates in live birds, and boluses.
2. Microplastics (<5mm): can be assessed from gastrointestinal contents in dead birds, live-bird regurgitates, faeces/guano and boluses.
3. Plastic-derived chemicals (additives): can be assessed in tissues/organs (e.g. liver, muscle, fat) in dead birds, and preen gland oil, stomach oil and plastic items recovered from live and/or dead birds. Additives can also be found in hatched and/or unviable eggs.
4. Plastic-adsorbed organic contaminants (e.g. PCBs -polychlorinated biphenyls- and POCs -organochlorine pesticides-): can be assessed in plastic items found in the gastrointestinal tract of dead birds or regurgitates in live birds.

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1. INTRODUCTION

Albatrosses and petrels (order Procellariiformes) are among the most threatened bird species in the world (Birdlife International 2018). As long-lived top predators, albatrosses and petrels can reflect the set of processes that affect their prey at lower trophic levels and can therefore be considered sentinels of ocean health (Furness 2003, Cardoso et al. 2014). Hence, they can be useful indicators of altered ecological processes and environmental conditions (Weimerskirch et al. 2003, Parsons et al. 2008, Grimaldi et al. 2014, Phillips et al. 2016).

Although bycatch is the main threat for most albatrosses and petrels (Phillips et al. 2016), these birds also face a range of other threats on land and at sea, including plastic ingestion and associated compounds (AC9 PaCSWG, Acampora et al. 2014, Wilcox et al. 2015, Roman et al. 2016, 2019). Procellariiformes are particularly susceptible to plastic ingestion, since they feed preferably on small prey on the waters' surface, where plastics tend to float and accumulate (Titmus and Hyrenbach 2011). The enormous amount of marine debris circulating in the world's oceans (Jambeck et al. 2015), the growing evidence of intentional or incidental ingestion by seabirds (Wilcox et al. 2015), and the lack of knowledge on the effects this may be having on the health of individuals, have highlighted the need for further investigation.

Ingested plastics can be classified by size (Barnes et al. 2009, GESAMP). For our purposes, macroplastics means >5mm and microplastics means <5mm. A variety of health effects are attributed to plastics exposure in marine animals. Macroplastics are most frequently associated with direct health effects when ingested due to their potential to cause injuries, suffocation or obstruct the gastrointestinal tract (Pierce et al. 2004; Phillips et al. 2010; Ryan 2016; Roman et al. 2019). However, the health effects of microplastics ingestion remain poorly understood (Rochman et al. 2014, Limonta et al. 2019, Fossi et al. 2020). Additionally, the accumulation of chemicals derived from plastic degradation (e.g. additives such as plasticizers and flame retardants) has also been documented in marine fauna (Tanaka et al. 2013, 2015; Fossi et al. 2012, 2014; Hardesty et al. 2015, Baini et al. 2017, Provencher et al. 2020). Most of these compounds are potentially toxic and are known to induce a broad variety of chronic and sub-lethal toxic effects, including endocrine dysfunction, immune response disruption, mutagenesis and carcinogenesis (Finkelstein et al. 2007, Teuten et al. 2009, Hirai et al. 2011, Fossi et al. 2018). Their accumulation over long periods of time (e.g. chronic leaching from plastic particles retained in the stomach) may affect the life cycle and reproductive success of species, potentially leading to long term harm at the population level (Finkelstein et al. 2007, Hardesty et al. 2015). Moreover, PCBs (polychlorinated biphenyls) and POCs (organochlorine pesticides) that have an affinity for organic and plastic particles, on which they tend to be adsorbed (Mato et al. 2002, Endo et al. 2005, Ríos et al. 2007) have also been reported in plastic fragments ingested by seabirds (Colabuono et al. 2010, Yamashita et al. 2011).

To assess the pervasiveness of plastic ingestion among seabirds generally it is important to quantify characteristics of plastic ingestion across a range of species (Avery-Gomm et al. 2016, Provencher et al. 2014) using standardized methods (van Franeker et al. 2011). Methods development for sampling and analysis of plastics is an important, emerging area of research and development in marine litter science.

During the ninth meeting of the Advisory Committee (AC9), the Population and Conservation Status Working Group (PaCSWG) noted the widespread intrusion of both macro- and microplastic in the diet and environment of seabirds and expressed concern about forecasts that this will increase. Considering that marine plastic initiatives are underway by others including the Convention on Migratory Species (CMS), the Commission for the Conservation

of Antarctic Marine Living Resources (CCAMLR) and the International Maritime Organization (IMO), the PaCSWG agreed that ACAP could contribute to this topic through various actions. One such action is the production of guidelines to assess the incidence of plastic ingestion in ACAP species. Thus, during PaCSWG4 and PaCSWG5 we provided a draft set of guidelines for consideration of the working group. Comments and recommendations have been incorporated in the current revised sampling guidelines to assess plastic ingestion (macro and microplastics as well as additives and adsorbed chemical compounds) with an array of sample type choices from live and dead birds and/or their immediate environment that should facilitate collection in diverse settings. Although we focus on albatross and petrel species, these guidelines and recommendations are generalizable to other taxa.

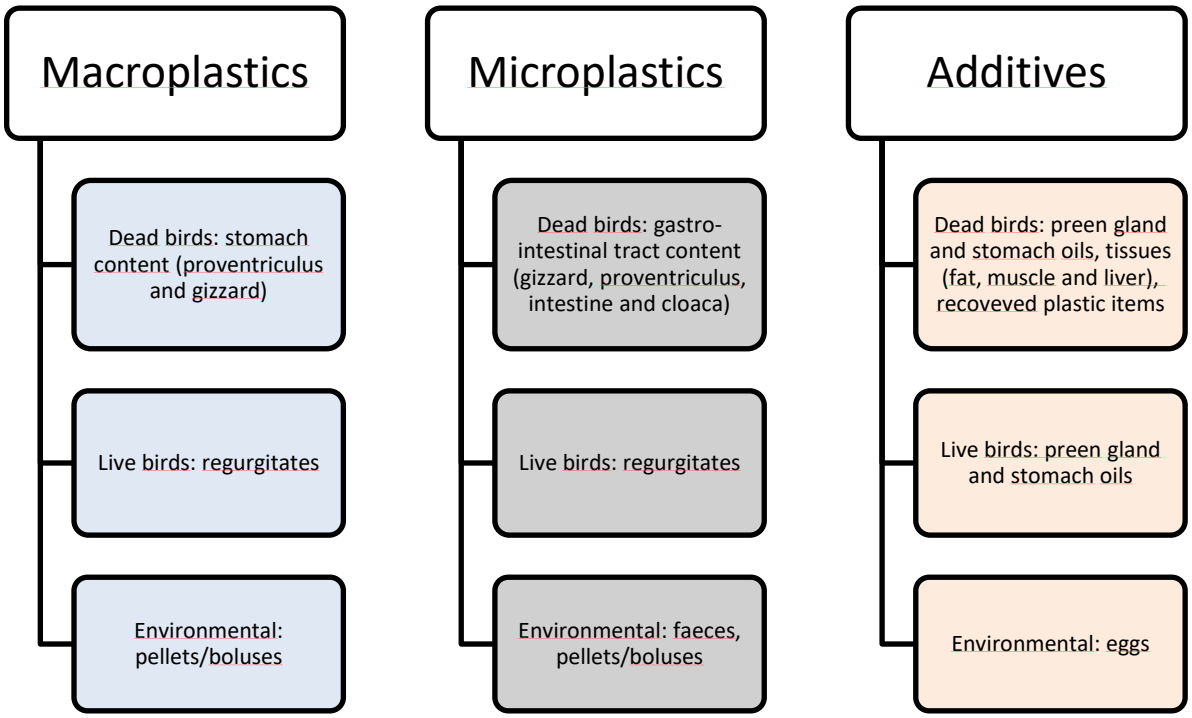
2. SAMPLE ACQUISITION AND STORAGE TO ASSESS PLASTIC INGESTION IN ACAP SPECIES

Sampling from dead birds (e.g. by-caught, beach-cast, at breeding sites or rehabilitation centers) can be performed on recently acquired or stored frozen carcasses. While freshness is a plus, carcass decomposition is less of a problem than scavenging, where important sections (e.g. digestive tract) might be missing. Sampling from live birds (e.g. rehabilitation centers or breeding sites) can be performed opportunistically, when handling birds for other purposes, or as targeted sampling for plastics investigation. Sampling live animals, however, requires specific training and skills as well as appropriate permits, and should therefore be restricted to personnel with the necessary expertise and approvals. Also, biases inherent to each sampling approach should be considered during study design.

Studies aiming to assess plastic additives or adsorbed contaminants must consider sampling within a controlled setting (e.g. lab or similar facility) to reduce contamination risk and enable having all proper sampling utensils and supplies easily at hand. Details on cleaning and sterilizing utensils for sample collection and storage are provided below in item 5. Under field conditions, and to the extent possible, materials must be single-use until they can be re-sterilized in order to avoid contamination. When sampling, contact with plastics, latex, etc. (e.g. gloves, bags, vials, syringes, others), should be avoided. Wearing nitrile gloves is recommended.

Whenever possible, we encourage collection of samples for immediate use as well as archival storage. If immediate interest is only a quick macroscopic assessment of plastic ingestion, it would still be ideal if the collection and storage of samples was done in such a way as to allow more dedicated complementary studies in the future (e.g. assessment of microplastics and/or additives). For this, however, collection, handling and storage need to follow strict procedures to avoid contamination and invalidation of often irreplaceable samples. In the sections dedicated to each plastic type we offer guidance for the simplest approach possible. Yet if samples are intended for immediate as well as future use, the more complex sterility processes will have to be followed (“clean” in text and tables implies heat-treated). When in doubt, handle all specimens with extra care and place in heat-treated aluminium foil prior to storage in other containers (e.g. sealable plastic bags).

IN ALL CASES: Wash hands thoroughly with soap and water prior to and after sample collection for personal protection.



Summary of sample types and sources for assessment of macro- microplastics, and additives. Adsorbed chemical compounds (e.g. PCBs) can be assessed in plastic items recovered from the gastrointestinal tract of dead birds or regurgitates in live birds.

2.1. Macroplastics

Sample type	*Sample source*	*Analytical method and references*	*Sample collection and storage*
Solid stomach content	Live bird (regurgitates)	Visual classification of plastic items: *Carey et al. 2011, Lavers et al. 2014, Provencher et al. 2014, Copello and Quintana 2003, Copello et al. 2008.*	- metal tray, forceps/tweezers, scissors, scalpel for dissection and cotton thread to tie the ends of the gastrointestinal tract. - put whole stomach or gastrointestinal tract or regurgitates in sealable plastic bag* and store in freezer for later transport/analysis.
	Dead bird (proventriculus and gizzard)	Visual classification of plastic items from the proventriculus and gizzard: *Colabuono et al. 2009, Jimenez et al. 2015, Ryan et al. 2016, Roman et al. 2016, 2019, Hyrenbach et al. 2017, Provencher et al. 2014, 2018, Van Franeker et al. 2011, Avery-Gomm 2020.*	
Pellets/boluses (indigestible items)	Environmental	Visual classification of plastic items in boluses: *Lindborg et al. 2012, Hammer et al. 2016, Hyrenbach et al. 2017.*	- forceps/tweezers - put boluses in sealable plastic bag and store refrigerated for transport. - store in freezer until visual analysis or dry and store in a dry and dark room until analysis.

***** *if chemical analysis (e.g. additives) will be performed, wrap stomach or GI tract (dead birds) in clean aluminium foil prior to storing in sealable plastic bag. In live birds, place regurgitates (solid and oil) in a cleaned glass container with aluminium foil under the lid (or use lids with PTFE liners). Alternatively, if only the solid content from regurgitates will be analysed, collect regurgitates in a cleaned metal or glass container and wrap retrieved solids in cleaned double aluminium foil prior to storing in sealable plastic bag.*

2.1.1. Gastrointestinal tract sampling

In dead birds, the gastrointestinal tract or stomachs (proventriculus and gizzard) can be recovered through dissection or during necropsy (for details, see Van Franeker 2004). Stomach contents from live birds can be collected in many species from adults and large chicks by spontaneous, voluntary regurgitation (Provencher et al. 2014). Water-offloading (lavage or flushing) or emetics are highly invasive and not recommended for this purpose only. Forced regurgitation can cause injury and mortality, and bias the sample obtained in live birds (Provencher et al. 2019). Note that the gizzard of Procellariiforms (except albatrosses) is separated from the proventriculus by an isthmus juncture where hard items can become lodged and are not easily regurgitated (Furness et al. 1985). Keep in mind that sampling techniques involving regurgitation rarely render a complete sample. For more details and special considerations, consult Provencher et al. (2019).

Regurgitates from live birds can be obtained by up-ending birds over a plastic bag, gently massaging the stomach and throat. In the case of dead birds, after dissection place gastrointestinal tract with its ends tied with cotton thread in a sealable plastic bag. Store samples frozen for later transport and analysis.

If chemical analysis (e.g. additives) will be performed, wrap stomach or GI tract (dead birds) in clean aluminium foil prior to storing in sealable plastic bag. In live birds, place regurgitates (solid and oil) in a cleaned glass container with aluminium foil under the lid (or use lids with PTFE liners). Alternatively, if only the solid content from regurgitates will be analysed, collect regurgitates in a cleaned metal container and wrap retrieved solids in cleaned double aluminium foil prior to storing in sealable plastic bag.

For macroplastics recovery and analysis, stomach content or regurgitates can be washed and sieved through a 5 mm mesh (or 1mm if you also want to identify microplastics) to facilitate separation of large items. Recovered items should be dried at room temperature (until constant mass) and stored until visual analysis. Suggested best practices for categorizing items and reporting results have been detailed by Provencher et al. (2017).

2.1.2. Environmental sampling

Boluses/pellets: regurgitated pellets of indigestible material (boluses) containing both debris and natural food items can be found in nesting colonies. Use clean forceps to place fresh intact feed-boluses individually in sealable plastic bags or double aluminium foil and store frozen until visual analysis (Colabuono et al. 2009, Van Franeker et al. 2011, Provencher et al 2014, 2018, Jimenez et al. 2015, Ryan et al. 2016, Hyrenbach et al. 2017).

For analysis, boluses should be dissolved with water and sieved through a 5 mm mesh (or 1mm if you also want to identify microplastics). Recovered items should be dried at room temperature (until constant mass) and stored until visual analysis. Suggested best practices for categorizing items and reporting results have been detailed by Provencher et al. (2017).

Note that although examining boluses is a useful, non-invasive sampling technique, comparisons among species are limited to the few species that produce boluses. Furthermore, it is unclear how much plastic is regurgitated with the boluses, and how much plastic remains in the birds or is excreted (Provencher et al. 2019).

2.2. Microplastics

Sample type	*Sample source*	*Analytical method and references*	*Sample collection and storage*
Faeces*[1]	Environmental	Visual analysis by microscopy of plastic items from faeces: *Gil-Delgado et al. 2017 (seabirds), Lusher et al. 2018, Nelms et al. 2019 (marine mammals).* Polymer type confirmation by ATR-FTIR spectroscopy: *Lusher et al. 2018, Nelms et al. 2019.*	- use disposable wooden spatulas to collect faeces in screw top vials or sealable plastic bags[2*] and store in freezer for later transport/analysis.
Pellets/ boluses (indigestible items)*[1]		Visual analysis by microscopy of plastic items in boluses: *Hyrenbach et al. 2017, Álvarez et al. 2018.* Polymer type confirmation by FTIR spectroscopy: *Alvarez et al. 2018.*	- forceps/tweezers - put boluses in sealable plastic bag or in cleaned double aluminium foil and store refrigerated for transport. -store in freezer until visual analysis or dry and store in a dry and dark condition until analysis.
Gastro-intestinal tract content	Dead bird (gizzard, proventriculus, intestine and cloaca)	Visual analysis by microscopy of GI tract content and later polymer confirmation and characterization by FTIR spectroscopy: *Lusher et al. 2015, 2018 (marine mammals), Avery-Gomm et al. 2016, 2018*, 2020 (*seabirds*). Visual analysis by microscopy of GI tract content: Van *Franeker et al. 2011, Provencher et al. 2018a*, *Lavers et al. 2019.*	- metal tray, forceps/tweezers, scissors, scalpel, for dissection and cotton thread to tie the ends of the GI tract. - put GI tract or regurgitates in sealable plastic bag[2], and store in freezer for later transport/analysis.
	Live bird (regurgitates)[1]	Visual analysis by microscopy: *Lusher et al. 2018.*	

[1] faeces, boluses and regurgitates are not appropriate to assess ingestion of plastic items <1mm because of the high levels of environmental contamination.

[2] if chemical analysis (e.g. additives) will be performed wrap stomach or GI tract (dead birds) in clean aluminium foil prior to storing in sealable plastic bag. In live birds, place regurgitates (solid and oil) in a cleaned glass container with aluminium foil under the lid (or use lids with PTFE liners). Alternatively, if only the solid content from regurgitates will be analysed, collect regurgitates in a cleaned metal or glass container and wrap retrieved solids in cleaned double aluminium foil prior to storing in sealable plastic bag.

2.2.1 Gastrointestinal tract sampling

In dead birds, the gastrointestinal tract (proventriculus, gizzard, intestine, cloaca) can be recovered through dissection or necropsy (for details, see Van Franeker 2004). This sample is recommended to assess microplastic ingestion, including items <1mm. Stomach contents from live birds can be collected from adults and large chicks by spontaneous regurgitation in many species (Provencher et al. 2014). Water-offloading (lavage or flushing), or emetics are highly invasive and not recommended for this purpose. Note that the gizzard of Procellariiforms (except albatrosses) is separated from the proventriculus by an isthmus juncture where hard items can become lodged and are not easily regurgitated (Furness et al. 1985). This can cause injury and mortality, and bias the sample obtained in live birds. Keep in mind that these sampling techniques rarely render a complete sample. For more details and special considerations, consult Provencher et al. (2019).

Regurgitate samples from live birds can be obtained by up-ending birds over a plastic bag, gently massaging the stomach and throat. From dead birds, after dissection place

gastrointestinal tract with its ends tied with cotton thread in a sealable plastic bag. Store samples in freezer for later transport and analysis (see 2.2.3).

If chemical analysis (e.g. additives) will be performed, wrap stomach or GI tract (dead birds) in clean aluminium foil prior to storing in sealable plastic bag. In live birds, place regurgitates (solid and oil) in a cleaned glass container with aluminium foil under the lid (or use lids with PTFE liners). Alternatively, if only the solid content from regurgitates will be analysed, collect regurgitates in a cleaned metal or glass container and wrap retrieved solids in cleaned double aluminium foil prior to storing in sealable plastic bag.

2.2.2. Environmental sampling

Fresh faeces and regurgitated feed-boluses or pellets can be collected to assess plastic ingestion (items ≤ 5mm - >1mm) in seabirds. Collection of these samples from within and near nests is a good option when working at breeding colonies. It is non-invasive and does not require handling birds. However, it is unclear how much plastic is regurgitated with the boluses or is excreted in faeces and how much plastic remains in the birds (Provencher et al. 2019). Thus, these samples are not recommended to quantify plastic ingestion.

a) **Faeces sampling**: Scoop faeces with disposable wooden spatulas. Place in sealable plastic bags or glass vials and freeze. Alternatively, collect faeces, dry at room temperature, weigh (until constant mass) and then freeze for later visual analysis (see 2.2.3) (Gil-Delgado et al. 2017, Nelms et al. 2019). Faecal collection can be combined with DNA studies to investigate both plastic ingestion and diet from the same sample (Nelms et al. 2019).

b) **Boluses:** regurgitated pellets of indigestible material (boluses) containing both debris and natural food items can be found in nesting colonies. Use forceps to place fresh intact feed-boluses individually in a plastic bag or cleaned double aluminium foil and store refrigerated for transport. Store in freezer or dried (until constant mass) and store in dry and dark condition until analysis (see 2.2.3) (Hyrenbach et al. 2017). Note that although examining boluses is a useful, non-invasive sampling technique, comparisons among species are limited to the few species that produce boluses.

2.2.3. Procedures for the analysis of microplastics

Analysis of microplastics, especially when targeting plastic particles < 1 mm, can be relatively complex and requires working in a controlled environment (e.g. lab with laminar flow cabinet), minimizing sample contamination. Specifics include working with filtered air and under a fume or under-pressure hood, keeping samples covered as much as possible, as well as using a pyramid glove box for certain steps of the process (Provencher et al. 2019). Lab staff should wear cotton lab coats possibly in an uncommon colour (e.g. pink or orange) for easy detection of fibres originating from lab clothes. Finally, environmental blanks should be used to quantify the risk of airborne microplastic sample contamination during processing. Here we provide general guidance for microplastics analysis, but detailed procedures and supplies needed can be found in mentioned references below.

If samples are not fluid, they can be poured into a container with distilled pre-filtered water for a few hours to hydrate before processing.

Step 1: removal of inorganic material (optional): 5 mol/L NaCl solution (density flotation) can be used to separate sand and grit from plastic items and prey items (Provencher et al. 2019).

Step 2: removal of organic material (<1mm items). Perform enzymatic (e.g. Proteinase K, Lipase), acid (e.g. HNO3, HClO4, CH2O2), alkaline (e.g. KOH and NaOH) or oxidizing digestion (e.g. H2O2) (Cole et al. 2014, Lusher et al. 2017, Provencher et al. 2018a, Lavers et al. 2019). Some protocols include incubation in a thermostatic bath to ensure complete decomposition. Note: Digestion protocols should only be used when necessary (<1mm items), and the solution and potential impact on the integrity of the sample (e.g., impacts on colour, mass, or degradation of certain polymer types) should be recorded. Typically, plastics >1 mm can be identified after filtration (step 3) without digestion protocols by an experienced observer.

Step 3: plastic particle extraction. Filter the samples. The mesh size selected determines the minimum size that is targeted for sampling. One-millimetre sieves are commonly used and recommended for microplastics (1-5mm) diagnosis. For smaller particles (<1mm) a second filtration under vacuum using equipment such as a glass Buchner filter with a microfiber filter (GF/D or alternative) is recommended. When large amounts of undigested organic material (e.g. bones) remain after filtering, density flotation (step 1) can be used to separate undissolved organic material from low density plastics which will float.

Step 4: visual and chemical identification of plastic particles. First, samples should be covered and air-dried for at least 24 h at room temperature or for a minimum of 12 h in a drying oven at 40 °C. For particles 1–5 mm, visual evaluation by stereo-microscope is usually performed (Van Franeker et al. 2011, Lusher et al. 2015, Avery-Gomm et al. 2016, 2018, Gil-Delgado et al. 2017, Provencher et al. 2014, 2018). Alternative approaches include staining with Nile Red, which makes plastic pieces fluoresce under blue light to facilitate identification (Maes et al. 2017). Also, chemical and physical characterization of recovered materials by spectroscopic techniques, such as Fourier-transform infrared (FTIR) and Raman spectroscopy, are particularly useful for confirm visual analysis of microplastics (Alvarez et al. 2018, Lusher et al. 2018, Nelms et al. 2019, Avery-Gomm 2020) and to identify particles in the 500–50µm and 50–1µm size, respectively (Käppler et al. 2016).

2.3. Plastic-derived chemicals (additives)

If possible, body samples (preen gland, tissues/organs) plus plastic items from stomach content should be collected from the same animal to link detection of chemical compounds in both, recovered plastic items and body samples. Despite limited knowledge on plastics GI transit time, additives such as plasticizers (phthalates) and some PBDEs (higher-brominated congeners) are metabolized relatively quickly, and thus are less biomagnified or not at all. Presence in body tissues should therefore reflect recent leaching (Tanaka et al. 2020).

Sample type	*Sample source*	*Analytical method and references*	*Sample collection and storage*
Preen gland oil	Live bird	- phtalates in preen gland oil (recent 3–6 months exposure): *Hardesty et al. 2015.*	- clean sterilized metal spatula - glass vial with aluminium foil under the cap (or use lids with PTFE liners). - alternatively, wipe the gland with a glass microfiber filter and save it in a cleaned aluminium foil envelope. - keep cool in field, then store in freezer for later transport/analysis.
	Dead bird	- phtalates in preen gland oil (recent 3–6 months exposure): *Hardesty et al. 2015.* - flame retardants (PBDEs) and UV stabilizers: *Tanaka et al. 2020*	- clean sterilized new scalpel blade and tweezers/forceps to dissect gland. - cleaned double aluminium foil to store dissected gland.
GI tract content (oil and plastic items)	Live bird (regurgitates)	- leaching of flame retardants (PBDEs) in stomach oil: *Tanaka et al. 2015.*	- place regurgitate (solids and oil) in clean glass container with aluminium foil under cap (or use lids with PTFE liners). - alternatively, collect regurgitates in cleaned metal or glass container and place retrieved solids in cleaned double aluminium foil prior to storing in sealable plastic bag.
	Dead bird	- flame retardants (PBDEs) and other additives in ingested plastics*: Tanaka et al 2013, 2015, 2019.* -leaching of PBDEs in stomach oil: *Tanaka et al. 2015*	- metal tray, clean sterilized new scalpel blade and clean scissors, forceps/tweezers for dissection and cotton thread to tie the ends of the GI tract. - wrap GI tract (dead birds) in cleaned double aluminium foil prior to storing in sealable plastic bag, then store in freezer for later transport/analysis.
Abdominal fat tissue and pectoral muscle	Dead bird	- phtalates and other additives in whale blubber and muscle: *Fossi et al. 2012, 2014.* - flame retardants (PBDEs) and other additives in bird fat tissue and muscle: *Tanaka et al. 2013, 2015, 2020, Commendatore et al. 2018.*	- metal tray, clean sterilized new scalpel blade and clean scissors, forceps/tweezers for dissection. - cleaned double aluminium foil and store in freezer for later transport/analysis.
Organs (e.g. liver)	Dead bird	- flame retardants (PBDEs) and other additives in bird liver: *Tanaka et al. 2015, 2020, Commendatore et al. 2018.*	- metal tray, clean sterilized new scalpel blade and clean scissors, forceps/tweezers for dissection. - cleaned double aluminium foil and store in freezer for later transport/analysis.
Eggs	Environmental	- flame retardants (PBDEs): *Jaspers et al. 2005, Polder et al. 2008, Braune et al. 2015, Commendatore et al. 2018.*	- cleaned double aluminium foil - keep cool in field, then store in freezer for later transport/analysis.

Note that contamination is a very important concern for identification and quantification of plastic additives. The use of properly cleaned utensils and supplies for sample collection and storage is essential for assessment of plastic-derived chemicals. If you lack capacity to have properly prepared materials at hand, you should disregard collecting samples for plastic chemical compound analysis.

2.3.1. Preen-gland oil sampling

1) To collect the preen gland in dead birds, simply use a clean, sterilized, new scalpel blade to excise the gland (help yourself with clean forceps/tweezers if needed), avoiding all contact with plastics, gloves, etc. Place the gland in clean double aluminium foil, label and store in freezer.

2) To collect preen gland oil from live birds, gently massage the preen gland at the upper base of the tail, then give a gentle squeeze to express a very modest amount of oil. Following Hardesty et al. (2015), carefully remove sterilized stainless-steel spatula from glass vial and swab over oil gland to pick up exudate. Return spatula to glass vial without touching any plastic. Make sure clean aluminium foil is placed over the top of the vial before screwing on the plastic lid (or use lids with PTFE liners). Alternatively, follow Yamashita et al. (2018) and wipe preen gland oil using a glass microfiber filter. Avoid contact with bird feathers. Store in a cleaned aluminium foil envelope.

2.3.2. Gastrointestinal tract sampling

In dead birds, the gastrointestinal tract can be extracted through dissection or during necropsy (for details, see Van Franeker 2004). Be careful in tying both ends to preserve oil and solid items in content. Stomach contents from live birds can be collected from adults and large chicks by spontaneous regurgitation in many species (Provencher et al. 2014). Water-offloading (lavage or flushing), or emetics are highly invasive and not recommended for this purpose only. Note that the gizzard of Procellariiforms (except albatrosses) is separated from the proventriculus by an isthmus juncture where hard items can become lodged and are not easily regurgitated (Furness et al. 1985). This can cause injury and mortality, and bias the sample obtained in live birds.

Regurgitate samples from live birds can be obtained by up-ending birds over a cleaned glass container, gently massaging the stomach and throat. Save content in container with screw-top lid, placing aluminium foil under the lid (or use lids with PTFE liners). Alternatively, if only the solid content will be analysed, collect regurgitates in a cleaned metal or glass container and wrap retrieved solids in cleaned double aluminium foil prior to storing in plastic bag. For dead birds, place gastrointestinal tract with its ends tied with cotton thread in a sealable plastic bag.

Store samples in freezer for later analysis (Tanaka et al. 2015). Open the digestive tract in the lab using a clean scalpel blade. Recovered solids from the gastrointestinal tract (see 2.1.1. for macroplastics and 2.2.3 for microplastics >1mm) should be wrapped in cleaned double aluminium foil and stored frozen until chemical analysis (Tanaka et al. 2013, 2015).

2.3.3. Fat, muscle and liver tissue sampling

After collection with sterilized scalpel, use clean forceps to place tissue in clean, double aluminium foil, and store frozen (Tanaka et al. 2013, 2015, 2020, Fossi et al. 2012, 2014, Commendatore et al. 2019).

2.3.4. Egg sampling

Plastic contaminants may transfer from the mother to the eggs (Jaspers et al. 2005, Polder et al. 2008, Braune et al. 2015, Provencher 2019, Commendatore et al. 2018). Hatched or unviable eggs can be collected from nests with nitrile gloves, wrapped in foil, and frozen until analysis.

2.3.5. Blanks and control samples

Collect two blanks while sampling live birds in the field (*environmental* blank) and/or dead birds in the lab (*dissection* blank). To collect a blank sample, wave a glass vial in the air (or any sample collection utensil such as metal spatula or glass microfiber filter), for one minute, without it touching anything. Place the lid, label with date, location, time, etc. and "*blank"*, as appropriate. In addition, one blank must be kept as a *transport* blank. This will not be opened but will be run with the other samples to ensure there is no contamination during submission of samples to the laboratory.

Due to the extensive presence of phthalates in many everyday items, even within clean laboratory environment, the presence of selected phthalates in collection and storage items (gloves, filter paper, needle, etc.) must be analysed to detect potential inadvertent contamination.

2.4. Adsorbed compounds

Sample type	*Sample source*	*Analytical method and references*	*Sample collection and storage*
GI tract content (plastic items)	Dead bird	- PCBs and OCPs adsorbed to ingested plastics: *Colabuono et al. 2010, Yamashita et al. 2011, Herzke et al. 2016, Provencher et al. 2018b.*	- metal tray, clean sterilized new scalpel blade and clean forceps/tweezers. - wrap stomach in clean aluminium foil and place in sealable plastic bag or cleaned glass container with aluminium foil under the lid. - store in freezer for later transport/analysis.
	Live bird (regurgitates)	- POPs adsorbed to ingested plastics: *Ríos et al. 2007.*	- collect regurgitate in cleaned glass container, place aluminium foil under cap (or use lids with PTFE liners). - Alternatively, collect regurgitate in cleaned metal or glass container and place retrieved solids in cleaned double aluminium foil prior to storing in sealable plastic bag.

Note that the use of properly cleaned utensils and supplies for sample collection and storage is essential for assessment of adsorbed contaminants. If you lack capacity to have properly prepared materials at hand, you should disregard collecting samples for these analyses.

2.4.1. Gastrointestinal tract sampling

In dead birds, the gastrointestinal tract can be extracted through dissection or during necropsy (for details, see Van Franeker (2004). Be careful in tying both ends to preserve oil and solid items in content. Stomach contents form live birds can be collected from adults and large chicks by spontaneous regurgitation in many species (Provencher et al. 2014). Water-offloading (lavage or flushing), or emetics are highly invasive and not recommended for this purpose only. Note that the gizzard of Procellariiforms (except albatrosses) is separated from the proventriculus by an isthmus juncture where hard items can become lodged and are not easily regurgitated (Furness et al. 1985). This can cause injury and mortality, and bias the sample obtained in live birds.

Regurgitate samples from live birds can be obtained by up-ending birds over a cleaned glass container, gently massaging the stomach and throat. Save content in container with screw-top lid, placing aluminium foil under the lid (or use lids with PTFE liners). Alternatively, if only the solid content will be analysed, collect regurgitates in a cleaned metal or glass container and wrap retrieved solids in cleaned double aluminium foil prior to storing in plastic bag. For dead birds, place gastrointestinal tract with its ends tied with cotton thread in a sealable plastic bag.

Store samples in freezer for later transport and post-processing. Open the digestive tract in the lab using a clean scalpel blade. Recovered solids from the gastrointestinal tract (see 2.1.1. for macroplastics and 2.2.3. for microplastics >1mm) should be wrapped in cleaned, double aluminium foil and stored frozen until chemical analysis (Ríos et al. 2007, Colabuono et al. 2010, Yamashita et al. 2011, Herzke et al. 2016, Provencher et al. 2018b).

3. SAMPLE LABELLING AND DATA COLLECTION

We recommend including the following information on sample labels:

- Three- or four-letter code - standard bird identifier (species initials, can use common or scientific name)
- Date _ yyyymmdd_ Xx type of sample (e.g. liver, preen gland – use initials)
- XX – number of sample (sequential for the same bird)

Example: **BBA_20150402_PG_01** which stands for: Black browed albatross, from 2nd April 2015, Preen gland, sample no 1.

When collecting several samples from the same animal, use same identifier but change sample type and number of sample.

Use permanent marker for labelling vials. If vial has no label, or when labelling aluminium foil, use paper or masking tape to create a label. When transferring samples always make sure that the labels are in good condition (re-label as necessary). For identification purposes it helps to have all samples from the same animal together. You can use clean, large aluminium foil sheets to wrap samples from the same individual (for plastic-derived chemical analysis), or Ziploc bags (other analysis).

In addition to recording types and numbers of samples collected from each individual animal, record location of sample collection as well as the person collecting the sample in your datasheets. This way, each sample will be linked to a site and responsible person.

4. SUPPLIES NEEDED FOR SAMPLE COLLECTION

1. **Glass vials** for preen gland oil, stomach oil, etc.: any clean vial can be used. A recommendation is using Corning "single use" centrifuge tubes which can be ordered from most lab suppliers. An example is Corning, product no 99502-10: 10ml (16x114mm) disposable glass screw cap centrifuge tubes with lids with PTFE liners. VWR catalogue no 33502-140. To reduce costs of PTFE lids, place aluminium foil (heat-threatened at 450ºC) under common plastic lids.
2. **Stainless steel spatulas:** To reduce costs, two-headed spatulas can be purchased and then cut in half. An example can be found at: http://www.sampling.com/stainless_micro_spatulas.html
3. **Disposable wooden spatulas:** also found as flat wooden tongue depressors.
3. **Aluminium foil:** commercial cooking aluminium foil.
4. **Microfiber filter wipe:** Whatman GFF, 47 mm diameter can be used to wipe the preen gland to collect oil for chemical analysis. https://www.sigmaaldrich.com/catalog/product/aldrich/wha1825047?lang=en&region=US
5. **Sealable plastic bags:** commercial sealable plastic bags (e.g. Ziploc)
6. **Glass container or jar**: Clean and sterilized according to 5 (below) with aluminium foil under the lid. Example: Corning, Pyrex®, catalog number: 1395-500.
7. **Metal trays**: for dissections or regurgitate collection.
8. **Dissection materials:** scalpel blades, scissors, forceps/tweezers. All must be clean and sterilized according to 5 (below).

Most sample analysis (e.g. microplastics, additives, etc.) require specific equipment (e.g. small mesh sieves, stereoscopic magnifying scopes, ultrasonic bath, centrifuge, lyophilizer, vacuum filtration equipment, etc.), reactants (e.g. solutions for digestion, solvents for extraction, etc) and/or precision techniques (gas chromatography-mass spectrometry, FTIR, Raman spectroscopy, etc). Refer to the literature references provided in tables and methods sections for specific information on analytical processes which are beyond the scope of these basic sample collection guidelines.

5. RECOMMENDATIONS FOR CLEANING AND STERILIZING UTENSILS AND MATERIALS

Properly cleaning glass vials, aluminium foil, metal trays and re-usable utensils (e.g. tweezers, scissors, scalpel) prior to sample collection and storage is essential. Because cleaning procedures require use of solvents and heating to high temperatures, consider contacting a local lab for help or resort to collaborators who may provide you with pre-cleaned materials and kits for the field.

Prior to sample collection, glass vials and reusable utensils should be washed thoroughly with distilled water and a brush. Rinse several times. Then, wash with solvents (3 times each): 1st methanol or acetone, 2nd dichloromethane (DCM), 3rd hexane. Alternatively, replace washes with solvents by heating the material to 450°C for 6 hours. Aluminium foil should be heated to 450°C for 6 hours.

To avoid contamination between individuals during sampling, use a new scalpel blade for each animal; reusable utensils should be washed thoroughly with running water and detergent and a brush and then rinsed with distilled water several times. To avoid contamination between samples taken from the same individual, 1st wash utensils with water, 2nd dry with paper towel, and 3rd rinse with alcohol.

6. CONCLUDING REMARKS

The aim of these guidelines is to present general and simple sampling options to assess plastic ingestion (macro- and microplastics, plastic additives and adsorbed compounds) in ACAP species. These protocols can be applied broadly both in the field by non-expert personnel (e.g. environmental and dead bird sampling), as well as by specialized personnel in the case of live birds or teams performing full necropsies in controlled settings.

At least four levels of analysis can be performed on the sample types suggested in the protocols above. With an increasing level of complexity (and generally increasing costs), it is possible to perform:

1) visual analysis to classify plastic items (macro- and microplastics >1mm) from stomach contents (Copello and Quintana 2003, Copello et al. 2008, Colabuono et al. 2009, Carey et al. 2011, Van Franeker et al. 2011, Provencher et al. 2014, 2018, Lavers et al. 2014, 2019, Jimenez et al. 2015, Ryan et al. 2016, Roman et al. 2016, 2019, Hyrenbach et al. 2017, Avery-Gomm 2020), boluses (Lindborg et al. 2012, Hammer et al. 2016, Hyrenbach et al. 2017), regurgitates (Lusher et al. 2018) and faeces (Gil-Delgado et al. 2017, Lusher et al. 2018, Nelms et al. 2019);
2) rapid-screening (detection and quantification) of microplastics (size limit of detection is defined by magnification and optical resolution) based on selective fluorescent staining using Nile Red, followed by density-based extraction, filtration and visual analysis (Maes et al. 2017);
3) chemical and physical characterization of nano-scale microplastics (also employed for confirmation after visual analysis of larger items in the order of mm) by spectroscopic techniques such as FTIR (500–50 µm) and Raman (50–1 µm) from boluses (Alvares et al. 2018), feaces (Lusher et al. 2018, Nelms et al. 2019), and stomach content (Lusher et al. 2015, 2018, Avery-Gomm 2016, 2018, 2020);
4) chemical analysis to identify and quantify specific plastic-derived (additives) compounds from preen gland (Hardesty et al. 2015, Tanaka et al. 2020), and stomach oils (Tanaka et al. 2015), plastic items (Tanaka et al. 2013, 2015), organs and tissues (Fossi et al. 2012, 2014, Tanaka et al. 2013, 2015, 2020, Commendatore et al. 2018), eggs (Jaspers et al. 2005, Polder et al. 2008, Braune et al. 2015, Commendatore et al. 2018);
5) chemical analysis to identify and quantify specific plastic-adsorbed compounds from plastic items (Colabuono et al. 2010, Yamashita et al. 2011);
6) chemical analysis to classify plastic fragments from chick regurgitated feed-boluses according to the resin codes used by the Society of Plastics Industry (Nilsen et al. 2014 - not included in tables).

There is consensus that assessing the pervasiveness of plastic exposure in seabirds requires adoption of standardized methods to facilitate cross-species comparisons, and to detect large

scale spatiotemporal patterns (van Franeker et al. 2011, Avery-Gomm et al. 2016, Provencher et al. 2014). At this time, we have purposely omitted inclusion of other sample types such as feathers (Adrogue et al 2019) and plasma (Leat et al. 2013, Roscales et al. 2016, 2019, Miller et al. 2020) since their value as indicators of plastics exposure is yet unclear due to information gaps in metabolism and/or due to increased complexities and high likelihood of contamination during collection and processing. These guidelines aim to provide selected methods and options based on the authors experience and should be re-visited frequently to incorporate newer, simpler and cheaper technologies as they become available and validated.

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