



Making use of apex predator sample collections: an integrated workflow for quality assured sample processing, analysis and digital sample freezing of archived samples

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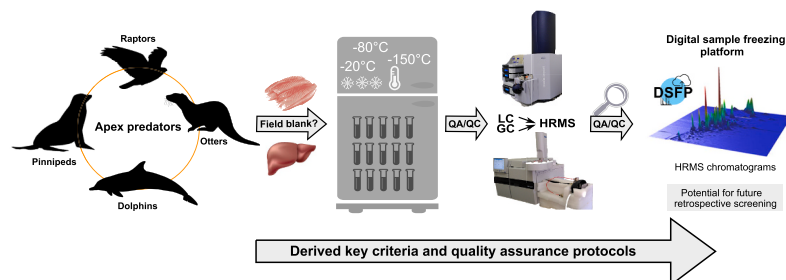
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HIGHLIGHTS

- Identifying regulatory needs for using predator monitoring data in risk assessment.
- Assessing the status quo of quality assurance measures in European sample collections.
- Workflow for quality assured sampling, processing, and analysis of archived samples.
- Focus on comprehensive chemical analysis such as non-target and suspect screening.
- Digital sample freezing of high-resolution chromatograms in databases.

GRAPHICAL ABSTRACT



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ABSTRACT

Using monitoring data from apex predators for chemicals risk assessment can provide important information on bioaccumulating as well as biomagnifying chemicals in food webs. A survey among European institutions involved in chemical risk assessment on their experiences with apex predator data in chemical risk assessment revealed great interest in using such data. However, the respondents indicated that constraints were related to expected high costs, lack of standardisation and harmonised quality criteria for exposure assessment, data access, and regulatory acceptance/application. During the Life APEX project, we demonstrated that European sample collections (i.e. environmental specimen banks (ESBs), research collection (RCs), natural history museums (NHMs)) archive a large variety of biological samples that can be readily used for chemical analysis once

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appropriate quality assurance/control (QA/QC) measures have been developed and implemented. We therefore issued a second survey on sampling, processing and archiving procedures in European sample collections to derive key quality QA/QC criteria for chemical analysis. The survey revealed great differences in QA/QC measures between ESBs, NHMs and RCs. Whereas basic information such as sampling location, date and biometric data were mostly available across institutions, protocols to accompany the sampling strategy with respect to chemical analysis were only available for ESBs. For RCs, the applied QA/QC measures vary with the respective research question, whereas NHMs are generally less aware of e.g. chemical cross-contamination issues. Based on the survey we derived key indicators for assessing the quality of biota samples that can be easily implemented in online databases. Furthermore, we provide a QA/QC workflow not only for sampling and processing but also for the chemical analysis of biota samples. We focussed on comprehensive analytical techniques such as non-target screening and provided insights into subsequent storage of high-resolution chromatograms in online databases (i.e. digital sample freezing platform) to ultimately support chemicals risk assessment.

1. Introduction

European sample collections archive a large variety of biological samples of which many can be used cost-effectively for chemical analysis and subsequent risk assessment once appropriate quality assurance and control (QA/QC) measures have been developed and implemented. Monitoring data from biota can play an important role for providing early warning of emerging contaminants, assessing the effectiveness of European chemicals legislations, prioritising chemicals for monitoring programmes, and the implementation of risk management options (see e.g. Movalli et al. (2017), Movalli et al. (2019) or Koschorreck et al. (2015)). Such data can therefore make an important contribution to the environmental monitoring called for under the EU Chemicals Strategy and proposed under the new European Partnership for Risk Assessment in Chemicals (PARC) (Dulio et al., 2020; PARC, 2020) and in support of the zero-pollution ambition of the European Green Deal (EC, 2021).

European initiatives have recognised the potential of biomonitoring data from apex predators and developed inventories for specific sample collections e.g. for apex predators in general (LIFE APEX, www.lifeapex.eu) or raptors in particular (European Raptor Biomonitoring Facility, Ramello et al. (2022)). Apex predators such as raptors, otters, and marine mammals have proven to be reliable sentinels for monitoring biomagnifying substances in their food webs due to their high trophic position, their well-known ecology, long history of ecotoxicological research and relatively long-life span (Badry et al., 2020; Megson et al., 2022; O'Rourke et al., 2022). Certain apex predators, e.g. raptors and pinnipeds, have suffered substantial population declines during the 20th century as a result of chemical pollution, and some continue to do so today (de Wit et al., 2020; Desforges et al., 2018; Shore and Taggart, 2019). Chemical monitoring of apex predators is therefore important not only for improving chemicals management, but also to obtain insights in contamination in food webs and species conservation. Population declines of top predators have been among the most tangible impacts of chemical pollution, and have driven public pressure to enact treaties aimed at reducing such pollution (Bierregaard et al., 2014; Blus et al., 1971).

Furthermore, the high trophic position of apex predators together with information on contamination levels in their prey may help to identify bioaccumulating chemicals and to derive field biomagnification factors (Swackhamer et al., 2009). Under current European chemicals legislations, information on bioaccumulating substances is usually derived under laboratory conditions using lower trophic level species such as fish according to OECD No 305 (OECD, 2012). In general, these tests are highly standardised and reproducible but have been shown to result in inaccurate predictions for exposures under field conditions as information on ecology, landscape and chemical mixtures is largely missing (Schäfer et al., 2019; Weisner et al., 2021).

Chemical monitoring and field data from apex predators can provide important information on whether or not a substance has the potential to bioaccumulate under field conditions and provide real-world exposure levels in wildlife that may be indicative for assessing the underlying food web as well as potential human exposures. Accordingly, the

European Commission calls for strengthening monitoring approaches in humans and ecosystems to improve the understanding of their impact and to act as EU early warning, which will further increase the number of chemicals measured in European compartments in the future (EC, 2020).

Up to now, wildlife monitoring data, especially from apex predators, are not routinely used by European Chemicals Regulators. Due to a lack of visibility of already available samples in European sample collections, monitoring campaigns using apex predators are usually considered to be very costly. However, in the case of apex predators, sample collections already exist and can be used (e.g. Ramello et al., 2022). In the case of apex predators, sample collections usually (and necessarily) archive samples from opportunistic sampling approaches, which increases variability in terms of species and sample matrices collected (vs. systematic/active monitoring campaigns). Specimens found dead by professionals and members of the public are brought into collections. Systematic sampling involving the culling of individuals is generally not feasible and ethically inappropriate as apex predators are typically protected species. Moreover, the purpose of sample collections can differ considerably among institutions, for many collections, contaminant monitoring is not a primary purpose.

QA/QC protocols are therefore needed in relation to sampling, sample processing and archiving, to assess the quality of samples for contaminant monitoring purposes. This is especially important for comprehensive chemical analysis using both liquid high resolution-mass spectrometry (LC-HRMS) and gas chromatography high resolution-mass spectrometry (GC-HRMS), which allow the determination of several thousand chemicals in each sample through wide-scope target analysis and suspect screening methodologies (e.g. in Badry et al. (2022b)). Such broad techniques require generic sample extraction protocols for extracting a large variety of chemicals with different physicochemical properties, which represents important progress since conventional targeted analytical methodologies are usually developed for a limited number of analytes (i.e. < 100). The acquired HRMS chromatograms can subsequently be stored in online databases, where they are accessible for retrospective screening using analytical information from substance lists (i.e. suspect screening) or without any prior information (i.e. non-target screening). Suspect and non-target screening represent feasible tools for detecting emerging contaminants as well as for identifying complex chemical mixtures that are currently not adequately addressed in European chemicals legislation (Drakvik et al., 2020; Hollender et al., 2019; Kortenkamp and Faust, 2018) while fully quantitative targeted analysis is particularly suited to monitor the impact of mitigation measures on wildlife exposures. Furthermore, the subsequent storage of HRMS data (digital sample freezing) allows the retrospective screening of chemicals that may be considered problematic in the future (Alygizakis et al., 2019; Hollender et al., 2019).

The presented work builds upon expertise and results generated during the LIFE APEX project (LIFE17 ENV/SK/000355), in which its partners analysed tissues from apex predators and selected prey species from the terrestrial (i.e. common buzzard), freshwater (i.e. Eurasian otter) and marine (i.e. marine mammals) environment in Europe using

target, suspect and non-target screening methods (Badry et al., 2022a). The current paper specifically presents a QA/QC workflow for biota samples from the collection stage to sample processing and preparation to high-resolution chemical analysis. Thereby, the paper aims to enhance the connection between researchers from different disciplines and chemical regulators who rely on high-quality data during chemicals assessments. Chemical information from apex predators can e.g. be used to assess the effectiveness of risk mitigation measures in case of trend data as well as in a weight of evidence approach to support the assessment of hazard endpoints, in particular bioaccumulation (Treu et al., 2022).

The first part of this paper investigates the current approach and future needs for promoting the use of chemical monitoring data from apex predators in chemicals regulations. In a second step, we then assess the status-quo of sample handling in European sample archives and develop key criteria for quality assured sampling, processing, archiving and shipment of biota/apex predator samples in Europe. In the next part, we present a workflow for generic sample extraction and chemical analysis using HRMS systems. The final part presents a workflow for digital sample freezing of HRMS chromatograms for retrospective suspect and non-target screening. Together, this provides a complete workflow from quality assured sampling of predator samples to digital sample freezing in online databases to ultimately strengthen the connection between science and regulation.

2. Monitoring data from apex predators: what is the perspective of European institutions involved in chemical risk assessment?

In May 2019, we issued an online questionnaire using *Google Forms* (<https://docs.google.com/forms>) to European institutions involved in chemical risk assessment and surveyed their experiences with apex predator data in chemical risk assessment (Table SI-1). Specifically, a profile on the current use of environmental monitoring data in chemical risk assessment was elaborated. Invitations to answer the questionnaires were sent out to representatives from the European Commission and European Chemical Agencies, academia, and industry via the CEFIC network (<https://cefic.org>). In total, 30 people responded to the questionnaire including the European Commission (DG Environment), 20 national competent authorities as well as 3 research and 3 industrial institutions. Not all respondents answered all questions, hence n varies for each question. Most participants indicated that they have experience in using chemical monitoring data for screening and/or prioritisation of chemicals (83%) followed by hazard assessments (48%), exposure assessment (41%) and evaluation of chemical risk mitigation measures (35%) (Figure SI-1, n = 29). The majority of the respondents used chemical monitoring data from abiotic matrices such as water, sediment and air (89%) as well as lower trophic level biota such as fish or sediment-dwelling organisms (79%), while only 29% indicated that they

were already using data from apex predators (Figure SI-2, n = 28). In total 90% of the respondents indicated that they are interested in using chemical monitoring data from apex predators of which 57% stated that they lack experience in assessing such data (Fig. 1, n = 30).

Obstacles for using chemical monitoring data from apex predators were indicated by 22 respondents. Answers were related to expected high costs, lack of standardisation and harmonised quality criteria for exposure assessment, data access, and regulatory acceptance/application. To support regulators towards the use of apex predator data, the next section, therefore, investigates currently applied sampling, processing and archiving procedures in European sample collections to derive key criteria necessary to allow for chemical monitoring and digital sample freezing from apex predator chromatograms.

3. From sampling to shipment: an integrated workflow for sample providers

3.1. Assessing the status-quo of sample handling in European sample collections

A separate questionnaire using the online tool *Umfrage Online* (<https://www.umfrageonline.com>) was issued to 27 European Environmental Specimen Banks (ESBs), Research Collections (RCs) and Natural History Museums (NHMs) in March/April 2019 on their use of protocols and quality assurance measures for sampling, processing, and archiving of apex predator samples. In total, five ESBs, five NHMs and five RCs returned the questionnaire (Table SI-2) resulting in 15 responses. In general, sample archives apply different sampling approaches (systematic and/or opportunistic) and storage conditions. ESBs usually rely on iterative active (systematic) sampling approaches, where specimen type, sampling region and sampling dates are fixed. As apex predators are protected species, invasive monitoring campaigns are impossible but collecting eggs, feathers or blood (if possible) might be feasible in some cases. Furthermore, many ESBs were founded in the 1980/1990's when populations of apex predators were still low, which is why many ESBs still focus on lower trophic levels species.

Today populations of many apex predators have recovered, which resulted in passive (opportunistic) sample collection schemes by NHMs as well as some RCs. However, the categorisation into the respective classes (ESBs, RCs, and NHMs) is not always strict as some overlaps between institutions exist. For example, one ESB is located at an NHM and partly relies on opportunistic samples, whereas most ESBs conduct long-term monitoring campaigns of a pre-defined set of sentinel species. For RCs, both active and passive sampling campaigns exist but the coverage of species and sample types was usually higher in RCs than in ESBs.

Despite these partial overlaps for some institutions, the survey revealed great differences in quality assurance measures between ESBs,

How do you perceive the benefit of chemical data from apex predators?

30 responses

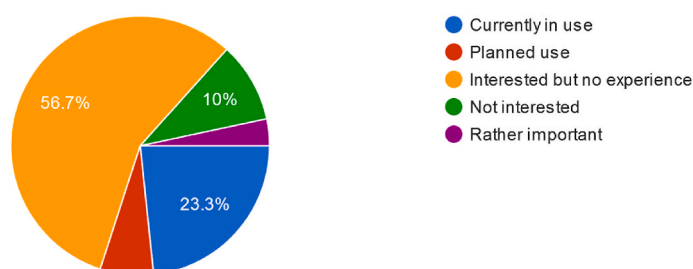


Fig. 1. Perception of the potential benefit of chemical monitoring data from apex predators among European institutions involved in European chemical risk assessment (n = 30 responses).

NHMs and RCs (Table 1). Whereas basic information such as sampling location and date as well as biometric data were mostly available across institutions, protocols to accompany the sampling strategy were only available in the majority of ESBs. The questionnaire by Ramello et al. (2022) on raptor collections in Europe reported that NHMs collect biometric data in 52% (n = 65) of the cases whereas RCs collected such data in only 35% of the cases (n = 23).

In our questionnaire, an implementation of sampling protocols was planned in several RCs and two RCs indicated that they are already using protocols. However, these protocols cannot always be applied due to the opportunistic sampling approach. No NHM indicated that there are formal protocols for the sampling procedure in place but one NHM indicated that they were planning to develop such a protocol.

Similar to sampling procedures, most ESBs also have protocols for sample processing that usually aim to minimise chemical (cross-) contamination. The majority of RCs have protocols for sample processing as well, but the intention of the documents varies depending on the research goal (e.g. avoidance of chemical vs microbiological cross-contamination). No NHM applied protocols for sample processing with regards to chemical (cross-) contamination but one NHM indicated a standardised sample processing procedure for DNA-based research. In the questionnaire by Ramello et al. (2022), 55% of the NHMs (n = 42) and 65% of the RCs (n = 20) use general protocols for preparing tissues for storage.

In our questionnaire, ESBs reported that contamination prevention is considered during all stages between sample collection and archiving by using e.g. materials of low contamination potential for sample processing (e.g., stainless steel and ceramics). However, specific measures vary across ESBs. Whereas some ESBs dissect animals in mobile laboratories under filtered air, others use a controlled atmosphere (clean room conditions). However, even for ESBs with a background in chemical monitoring, the use of appropriate field blank samples is not obligatory. In laboratories investigating environmental contaminants, laboratory blank samples are often routinely applied (especially when target analytes are personal care products or broadly used industrial chemicals). Some ESBs, on the other hand, use samples from pristine or near-natural regions that are analysed along with samples from anthropogenically impacted areas to help identify potential problems from contamination during sampling/processing. Furthermore, one ESB stated that procedures are currently implemented to minimise chemical contamination, which involves the identification of possible contamination sources by implementing suitable blanks in the workflow. In

Table 1

Summary of quality assurance/control measures indicated in the questionnaire for environmental specimen banks (ESBs), research collections (RCs) and natural history museums (NHMs). ✓ indicates that information was available, (✓) indicates that information was available in most cases, (X) indicates that information was not available in most cases, X indicates that no information was available.

	ESBs	RCs	NHMs
Basic information (sampling location & year, biometric data)	✓	✓	✓
Use of protocols on sampling strategy	(✓)	X	X
Use of protocols on sampling procedures	(✓)	(X)	X
Use of protocols on sample processing	(✓)	(✓)	X
Sampling, processing and archiving conducted or supervised by trained experts with background in chemical monitoring	✓	(✓)	X
Use of protocols on contamination prevention from external chemicals	✓	X	X
Samples maintained a cold chain after sampling and continuing during processing	✓	(✓)	(X)
Available documentation of sampling and processing of archived samples	✓	(✓)	(X)
Availability of additional information characterising the samples (e.g. age, sex, trophic position, data from chemical/molecular-biological analysis)	(✓)	(✓)	(X)

general, RCs and NHMs showed interest in advice to consider such quality assurance aspects in the future but specific measures are currently not in place.

Almost all participating ESBs implemented cold chains prior to archiving the samples and biota samples are frozen either directly in the field or shortly after sampling. In RCs, animals from opportunistic sampling campaigns are usually maintained in cold chains upon arrival of the carcasses in their facility. A special case may be bird eggs from (protected) apex predator species, which may not get frozen until weeks-months after collection from the nest. Not all NHMs are freezing samples or can maintain an uninterrupted cold chain for the samples. In general, archiving temperature is mostly $-20\text{ }^{\circ}\text{C}$ (max. $-15\text{ }^{\circ}\text{C}$) and $-80\text{ }^{\circ}\text{C}$ (max. $-70\text{ }^{\circ}\text{C}$) across institutions, whereas only two ESBs and one RC use a storage temperature of $-150\text{ }^{\circ}\text{C}$ or lower in the inert gas phase of evaporating liquid nitrogen. These results are in line with the questionnaire issued by Ramello et al. (2022), which reported that NHMs and RCs store samples mainly at $-20\text{ }^{\circ}\text{C}$ once the samples arrive at the institution.

ESBs generally have electronically available documentation of sampling and processing including metadata that is usually available upon request. Several RCs also implemented documentation of sampling and processing in databases or spreadsheets. In contrast, not all participating NHMs have complete documentation of sampling and processing of archived samples.

The last question in the questionnaire was related to the availability of additional information on biota samples that may improve the data evaluation such as age, sex, trophic position or data from prior chemical/molecular-biological analysis. Some ESBs record additional information such as necropsy reports, geographical information about the sampling sites as well as analytical results (contaminants, stable isotopes as proxy for trophic position, fat content). RCs store additional information as well (e.g. diet, genetics or parasite load) but the extent of information differs between collections and seems to be dependent on the specific research question. For NHMs, the extent of available information apart from the basic information (see above) varies as well. Only one NHM indicated that sex, age, and geographical data of the sampling region are recorded with each specimen along with results from analytical measurements.

In summary, the majority of participating ESBs reported that potential contamination of the samples during sampling and processing is addressed in routine operation and appropriate measures are taken to prevent such issues. Most ESBs indicated that their staff has long-term experience in sample handling and, in some cases, has a background in chemical monitoring. In some RCs, expertise on chemical monitoring is available and, in some cases, protocols on how to avoid contamination are implemented. In most cases experienced staff are involved in sampling, processing, and archiving in RCs to ensure consistent operations. Some RCs did not consider cross-contamination issues, especially if the samples are shipped to an external laboratory for chemical analysis but are becoming aware of them. NHMs participating in the survey often seemed less aware of potential contamination during sampling and processing, which may be related to the fact that chemical monitoring is not their primary focus. Therefore, in many cases, no specific measures are in place to avoid contamination during processing. However, it has been shown that NHMs receive the majority of opportunistically collected predator samples (Ramello et al., 2022) but lack protocols on sample handling and processing for chemical analysis. In contrast, ESBs usually sample biota from lower trophic levels using established quality assurance protocols for chemical analysis. Therefore, ESBs, RCs and NHMs are complementary to each other and a closer exchange between communities is expected to substantially increase the availability of high-quality apex predator samples.

3.2. Developing key indicators for assessing the quality of apex predator samples

Based on the questionnaire on the use of protocols and quality assurance measures for sampling, processing and archiving we derived key indicators for assessing the quality of apex predator samples from different archive types in Europe (ESBs, RCs, and NHMs). The development of such indicators is crucial for the interpretation of detected chemicals for which there is a potential for cross-contamination during sampling, processing and storage, from substances, such as personal care products (e.g. parabens or fragrances), veterinary antibiotics and analgesics or frequently used industrial chemicals in laboratories (e.g. plasticisers, flame retardants, etc). Such cross-contamination issues have also been raised in other contexts e.g. in relation to genetic analysis, and chemical contamination of food (Ballenghien et al., 2017; Rather et al., 2017). For example, when amplifying small amounts of genetic material by polymerase chain reaction (PCR), cross contamination with foreign genetic material frequently occurred, in particular for samples that were shipped on the same day to a laboratory (Ballenghien et al., 2017). Interestingly, sample-to-sample contamination with DNA was identified as the most problematic issue rather than external environmental contamination (Ballenghien et al., 2017) but this may be because molecular biology laboratories always work under clean air conditions using sterile equipment, which is not always the case for biota archives (see above). Both PCR and HRMS can be susceptible to trace contamination in the laboratories, which is why specific quality assurance measures are required if subsequent analyses are used to confirm the environmental exposure of biota to a chemical. Chemical contamination has also been recognised as an important concern for food due to environmental pollution and cross-contamination from food processing or migration from packaging material (reviewed by Rather et al. (2017)). An effective way to investigate the degree of contamination during sampling, processing and archiving is the use of field and laboratory blanks. In the case of biota sampling, a field blank could represent uncontaminated tissue material (similar to the sampled organs) that is brought to the field and then treated exactly the same way as the samples (exposure/processing on-site, processing and analysis in the laboratory). By this means possible sources of contamination during sampling, processing and sample containers may be identified during later analysis. However, such measures are currently not implemented in European sample collections, although exceptions might exist. As pragmatic means, biota samples from remote or pristine regions are often analysed in comparison to samples with higher burdens to identify possible cross-contamination issues.

Due to these current shortcomings in the chemical analysis of opportunistic samples, we established key indicators for assessing the quality of samples for environmental monitoring of chemicals based on the results of our questionnaire. These indicators are summarised in Table 2 and refer to obligatory minimum information (in bold) provided for sampling (e.g. traceable sample codes, sampling date & location), processing (e.g. dissection, homogenisation, pooling), and archiving (e.g. whole organism vs certain organs, date of freezing, storage temperature). The availability of such information is considered to significantly improve the quality assurance and interpretability of detected chemicals that have a high risk of external or laboratory contamination. Furthermore, additional information on potential deliberate veterinary treatments (e.g. euthanasia or treatment with antibiotics and analgesics) prior to death is considered to be important as high concentrations of antibiotics such as enrofloxacin ($>1000 \text{ ng g}^{-1}$ wet weight) have been detected in livers of European raptors (e.g. Badry et al. (2021)). A summary of derived key indicators for QA/QC indicators for all sample handling stages from collection, processing, and archiving to the analytical parts and digital sample freezing can be found in Fig. 2. Previous workflows, e.g. for analysing raptor tissues, described standard operation procedures for sample processing and give recommendations on sample containers (e.g. polypropylene, glass) as well as on transport

Table 2

Indicators for assessing the quality of apex predator/biota samples and their suitability for environmental monitoring studies. Key indicators are designated in bold.

	Indicators for sample processing
Collection/sampling stage	<ul style="list-style-type: none"> • Unique sample code/designation • Species name (if available, with information whether it is a sedentary or migrant species) • Date of sampling/finding • Sampling approach (opportunistic vs. systematic) • Location (preferentially geo-coordinates) • State of autolysis and, if possible, estimated time of death • Biometric data: e.g., weight, size/length, sex, age (juvenile/adult), cause of death • Information whether an individual was euthanised or received medical treatment prior to death
Processing stage	<ul style="list-style-type: none"> • Description of handling procedures (e.g., examination, organs dissected) • For preparation of homogeneously pooled samples: information on number of individuals, sampling year, amount of each individual used, age, and sampling region should be provided • Date of each processing step • Description of homogenisation of tissues (if applicable)
Archiving stage	<ul style="list-style-type: none"> • Date of freezing • Storage temperature ($-20 \text{ }^{\circ}\text{C}$, $-80 \text{ }^{\circ}\text{C}$, $-150 \text{ }^{\circ}\text{C}$) • Storage of whole organism or only certain organs • Amount of sample available • Material of sample packaging (e.g., glass bottles, aluminium foil)
Potential additional measures	<ul style="list-style-type: none"> • Availability of field-blank samples • Laboratory blanks • Information whether the sample is a biological hazard • Information on any materials that were in direct contact with the sample material/specimens during sampling or processing • Fat content of tissue • Water content of tissue • Trophic position (e.g. indicated by stable isotope analysis) • Data from previous (chemical) analyses

conditions and required sample quantities for selected target analysis (see Espín et al., 2020).

3.3. Shipping samples within the European Union

When sending samples of protected species among member states within the European Union, several considerations need to be made to comply with the European and international legislation and to ensure fast delivery of frozen samples within 24 h. Many apex predators are listed in Annex A or B of Regulation (EC) No 338/97, which is the implementation of the Washington Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in the European Union. For sending protected species, a sending institution (e.g. a sample collection) needs to have a CITES permit, which states (1) that the samples originated from the stated European country, (2) that the sending institution has the right to sample them, and (3) that the institution is allowed to ship them in the EU for scientific purposes. Such permits are usually available at institutions dealing with samples from endangered/protected species but can alternatively be requested at the national CITES management authority. A copy of this permit must be easily accessible on the outside of parcels in case of custom controls to ensure rapid onward transport. Furthermore, the receiving institution (e.g. an analytical laboratory) must be allowed to receive samples of species listed in Annex A or B of Regulation (EC) No 338/97. In some cases, a receiving institution might also be asked by a sending institution to provide their registration number in accordance with Regulation (EC) No 1069/2009, before sending the samples. The regulation refers to applied health rules for handling animal by-products that are not

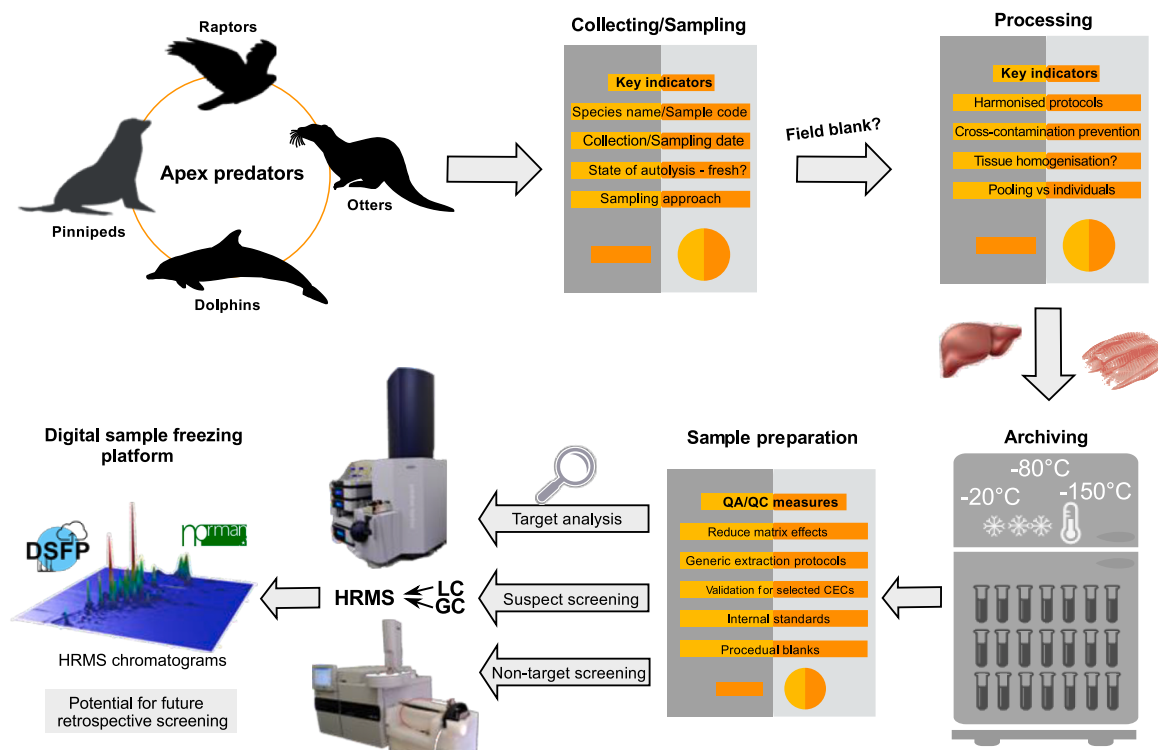


Fig. 2. Summary of an integrated workflow for quality assurance and control measures during sampling, processing, archiving, sample preparation and digital sample freezing of archived biota/predator samples.

intended for human consumption.

In addition to a CITES permit, a shipment must be accompanied by a “Non-Hazardous Content Declaration”, which consists of a brief statement that the samples are not harmful or contagious. This document must be placed outside of the parcel and is usually requested by receiving institutions as well as by shipping companies since e.g. avian influenza or rabies outbreaks in a sampling region may exclude a sample from shipment. A detailed step-by-step procedure for sending samples (wet weight) for species listed in the CITES Annexes can be found in the supplementary information (SI-3).

4. Sample preparation

For biomonitoring studies based on wide-scope target, suspect and non-target screening methodologies, the presence of thousands of chemicals with different properties is simultaneously investigated. Thus, there is an urgent need to develop and validate generic sample preparation protocols (as much as possible non-selective for a specific group of organic compounds), in order to capture a broad range of contaminants present in the investigated biota matrix. The analysis of such extracts allows in principle the retrospective screening of any new potential compound of interest. The choice of sample preparation method plays a key role in the sensitivity and selectivity of the applied methodology and thus, strongly affects the screening results.

Currently, harmonised methods for the extraction of contaminants from environmental matrices to be used for HRMS screening purposes have not been established yet, despite the efforts being made towards this direction via the implementation of HRMS interlaboratory studies by the NORMAN network. For example, lyophilisation in the sample pretreatment process has many potential advantages for overall data quality. In addition to enhancing the sensitivity of the applied method, it also facilitates homogenisation, which improves the precision of the results and allows for more accurate measurements on a small sample amount, which in the case of biota samples can be a limiting factor. In environmental sample analysis, the extraction of contaminants from

biota matrices is very challenging considering their high complexity due to the elevated levels of lipids, proteins and other biological compounds that may interfere with the compounds of interest (Hajeb et al., 2022). Therefore, additional purification steps must be incorporated in the sample preparation process to remove interfering matrix components and reduce matrix effects to result in more sensitive analytical methods. However, potential losses of analytes of interest should also be evaluated and considered during the method development. The best practice to avoid contamination during the sample preparation is by automating certain aspects of the process. For example, the automated extraction of the contaminants from biota matrices using pressurised liquid extraction (PLE) can considerably reduce the risk of human error and cross-contamination of samples and increase the reproducibility of the process (Vazquez-Roig and Picó, 2015).

Since suspect and non-target screening methodologies rely upon the identification of suspected or unknown compounds for which reference standards are not available, performing validation for a high number of contaminants may be extremely time and cost-consuming. The best adopted practice is to perform a smart validation of the developed methodology for a selected number of the contaminants included in the wide-scope target list (Gago-Ferrero et al., 2020; Gil-Solsona et al., 2021). The validation dataset should be selected in a way to ensure the representativeness of the whole target list, taking into account the physicochemical and analytical characteristics of the compounds (such as logP, functional groups, ionisation polarity and classification) (Gago-Ferrero et al., 2020). During the sample preparation, it is essential to use internal standards to correct reproducibility issues among the samples of the same or different batches and variabilities in instrumental parameters such as injection volume and MS sensitivity and ensure sufficient recovery of the contaminants from the analysed matrix. Therefore, internal standards, and especially stable isotopically labelled compounds, are highly recommended for reliable and accurate quantitative results in target analysis. Ideally, the respective isotopically labelled compound should be used for quantifying each contaminant. However, the cost of isotopically labelled compounds is high, their

commercial availability limited, and compounds present in a sample are screened by a database of thousands of contaminants that are not a priori known. Therefore, a mix of available isotopically labelled compounds of multi-class contaminants is usually used. Moreover, internal standards are also used in suspect and non-target screening workflows to minimise the effect of running batch (sequence of injections relating to each sample) and increase comparability between samples analysed on different days (Dürig et al., 2022; Nikolopoulou et al., 2022).

Although the purpose of generic sample preparation protocols is to extract a large number of compounds with a wide variety of physico-chemical properties, two protocols need to be followed to efficiently recover both polar to semi-polar compounds (LC-amenable) and non-polar, volatile and thermostable contaminants (GC-amenable compounds), using compatible solvents for extraction and solid phase extraction sorbents for further purification of the final extract (Alygizakis et al., 2022; Badry et al., 2022b). During the sample preparation of each batch, QC samples should be also prepared by the same method. Procedural blank samples are used to trace any unintentional contamination during the processing of the samples. To assess the recovery of the analytes and matrix effects spiked samples with a mixture of known contaminants should also be prepared and analysed. Certified reference materials (CRMs) with documentation of metrological traceability are currently not available for emerging contaminants. However, CRMs of relevant biota matrices for regulated persistent organic pollutants (POPs) should be included in the target database to evaluate the accuracy (trueness and precision) of the applied methodology.

5. Instrumental analysis

Apart from the regular system maintenance, a thorough QA/QC protocol should be followed during instrumental analysis to assure the separation efficiency of the analytes of interest and the good operation of the HRMS system.

After cleaning the source, transfer tube and cone, the sensitivity, mass accuracy, resolution and precision of the mass analyser should be monitored and compared to installation values. For this purpose, a system suitability protocol is recommended to be followed before analysing a batch of samples to make sure the system meets vendor's performance specifications (Caballero-Casero et al., 2021). The sequence of analysis is suggested to start with a QC sample (known sample, most preferably a mixture of standard solutions) to assess the system performance by evaluating the retention time, peak shape, chromatographic resolution, full width at half maximum (FWHM), background noise, mass accuracy and sensitivity, against well-defined pass and fail criteria and trigger a troubleshooting workflow if needed (Broadhurst et al., 2018). Each biota sample should run twice (in consecutive injections); in combined acquisition modes of full scan MS with (a) Data Dependent (DDA) and (b) Data Independent (DIA) MS/MS spectra, in order to record data needed for both targeted and untargeted screening workflows

(Menger et al., 2020). For a reliable quantitative analysis, blank solutions (instrument blanks) are measured after a sample analysis to monitor and reduce possible memory effect phenomena (or carry-over of analytes). A mix of known analytes (Retention Time Index (RTI) calibrant substances) can also be used to assess the stability of retention time during instrumental analysis and to enable future confirmation of suspect/non-target contaminants by different chromatographic systems/laboratories (Aalizadeh et al., 2021). A QC sample is recommended to run every 10–15 injections to ensure the good operation and high sensitivity of the system. Before starting the screening of the chromatograms, the sensitivity of internal standards in each sample is tested to assure satisfactory recovery and proper injection of the extracts into the chromatographic system. The main analytical practices and QA/QC steps that should be followed during the sample preparation and instrumental analysis to ensure the data quality of HRMS results are summarised in Fig. 3. Further information on the instrumental analysis can be found in SI-4.

6. Detecting signs of potential contamination

During the data processing and extraction of results, it is crucial to identify and trace potential contamination peaks. The task of identifying the source of contamination can be very challenging, considering that every point on a sample's path from initial collection to final analysis is a potential entry point for external contamination. An external contaminant can be a target analyte, a compound that co-elutes with the target compounds, or a compound not included in the list of analytes of interest. The external contamination mainly results in additive effects, meaning that the measured total analyte concentration is the sum of contributions from the sample and the contamination source. Furthermore, external contamination may result in the formation of chromatographic peaks that are coming solely from contamination and do not exist in the analysed samples. Although even when taking all precautionary measures during the sample preparation, following good laboratory practices, and instrumental analysis, the complete elimination of all external contamination peaks cannot be completely avoided. Blank samples (in duplicates or triplicates), including instrument (or system) blanks, method blanks and field blanks, can be powerful indicators for potential external contamination, as they are indispensable for checking the quality of reagents and materials used during the sample preparation and the instrumental analysis (EPA, 2014; Schulze et al., 2020). Furthermore, they can indicate possible sources and routes of contamination from the time of sampling (Boyd et al., 2008). The most common background contaminations encountered in MS are polyethylene glycol, polypropylene glycol, phthalates and other plasticisers, organic solvent clusters, solvent modifiers, fatty acids, and siloxanes. MS system contaminants will be detected in all samples, as well as in instrument blank injections. These blanks are analyte-free analytical reagents and provide a measure of the instrument system's

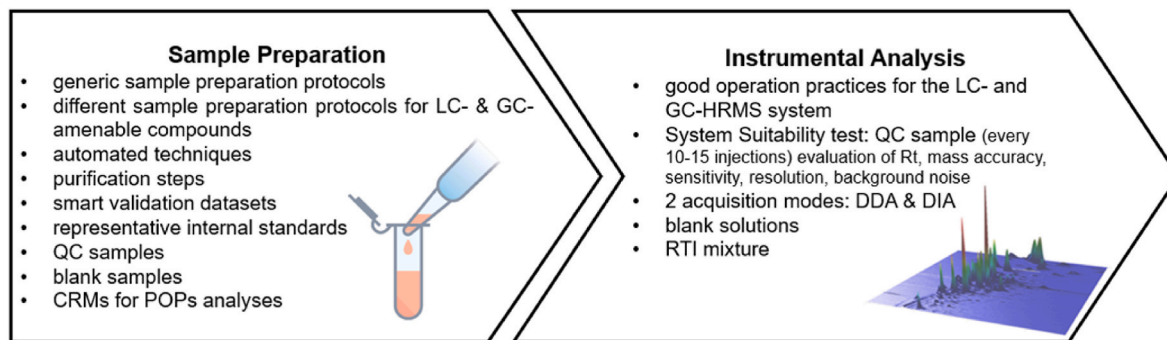


Fig. 3. The main analytical practices and QA/QC steps that should be followed during the sample preparation and instrumental analysis to ensure the data quality of HRMS results. The following abbreviations were used in the Figure; LC: Liquid Chromatography, GC: Gas Chromatography, QC: Quality Control, CRMs: Certified Reference Materials, POPs: Persistent Organic Pollutants, DDA: Data Dependent Acquisition, DIA: Data Independent Acquisition, RTI: Retention Time Index.

background noise and potential contamination. Method blanks (reagent blanks) are prepared at the same time as a batch of samples is prepared by the same method, to assess any external contamination which might have been introduced during the sample preparation of the final extracts for analysis (Caballero-Casero et al., 2021). The analysis of field blanks (uncontaminated biota tissue) could provide valuable information on contamination since they incorporate all potential sources, from sampling to archiving, processing and analysis. In the case of biota samples, and especially for wildlife species, uncontaminated tissues may not be available or guaranteed, therefore an alternative low contaminated sample (pseudo-blank) may be used. However, as previously mentioned, currently only a limited number of ESBs consider using samples of low anthropogenic impacted areas as field blanks in environmental monitoring studies. The detection and quantification of contamination that arises from blank sample analysis are essential since they may affect data quality. When quantifying the analytes of interest, the signals detected in blank samples should be subtracted from the respective signal of the samples. As a consequence, only the samples having significantly higher levels compared to the external contaminations will be reported as positive for the presence of the compound and the method detection limit should be adjusted based on method blanks (EPA, 2014).

7. Digital sample freezing for suspect and non-target screening

The physical specimens existing in the ESBs, RCs and NHMs contain chemical information and added value that can be harvested only if they are analysed by HRMS and the data is digitalized. Digital archiving of the HRMS data is of particular importance because it can be used for future retrospective suspect screening efforts upon specific contaminant requests from researchers, environmental agencies, and policy makers. HRMS data contains a wealth of unexploited information about the occurrence of chemicals in the sample of interest. For example, the NORMAN Digital Sample Freezing Platform (DSFP) represents a repository to safely store the digitalised biota samples to assure the longevity of the data (Alygizakis et al., 2019). NORMAN Association is an independent network of researchers (Slobodnik and Dulio, 2014) that

has established its database system for contaminants of emerging concern (Dulio et al., 2020). The DSFP is an indispensable part of the NORMAN Database System (NDS) and is available at www.norman-data.eu. The DSFP prototype was firstly presented at the NORMAN General Assembly in 2016. Afterwards, it was tested rigorously by a core group of experts. The purpose of the testing was to investigate harmonisation in uploading HRMS data to the DSFP from all HRMS vendors and all possible data acquisition methods. A technical guide was created (NORMAN, 2019), which allows digital archiving and subsequently automated retrospective suspect screening for thousands of contaminants (e.g. da Silva et al., 2021; Mascolo et al., 2019; Rostkowski et al., 2019) included in the chemical space of interest (NORMAN Network et al., 2020). The DSFP integrates many non-target screening advancements produced by other NORMAN activities such as the normalisation of the retention time dimension of the HRMS data using a set of calibrant substances (Aalizadeh et al., 2021), semi-quantification without reference standards, chemical domain applicability (Alygizakis et al., 2022) and knowledge gained within pan-European collaborative trials (NORMAN, 2022). The DSFP complies with the Findability, Accessibility, Interoperability and Reusability (FAIR) of data principles, which is a prerequisite for European projects. In total, 198 LIFE APEX samples from top predators and potential prey species enriched the collection and the spatial distribution of environmental samples in Europe (Fig. 4). It is worth highlighting that because of the LIFE APEX project, biota samples have become the second most prevalent group of environmental samples after surface water samples in the DSFP. The LIFE APEX data will play a critical role in chemicals management and the proposal of new chemicals for regulatory actions.

8. Outlook for future chemical monitoring to support risk assessment

Chemical data from biota including apex predators can provide important information about chemical exposures and the occurrence of chemical mixtures in the environment. Experiences from wildlife research programmes, e.g. LIFE APEX and ERBFacility revealed that

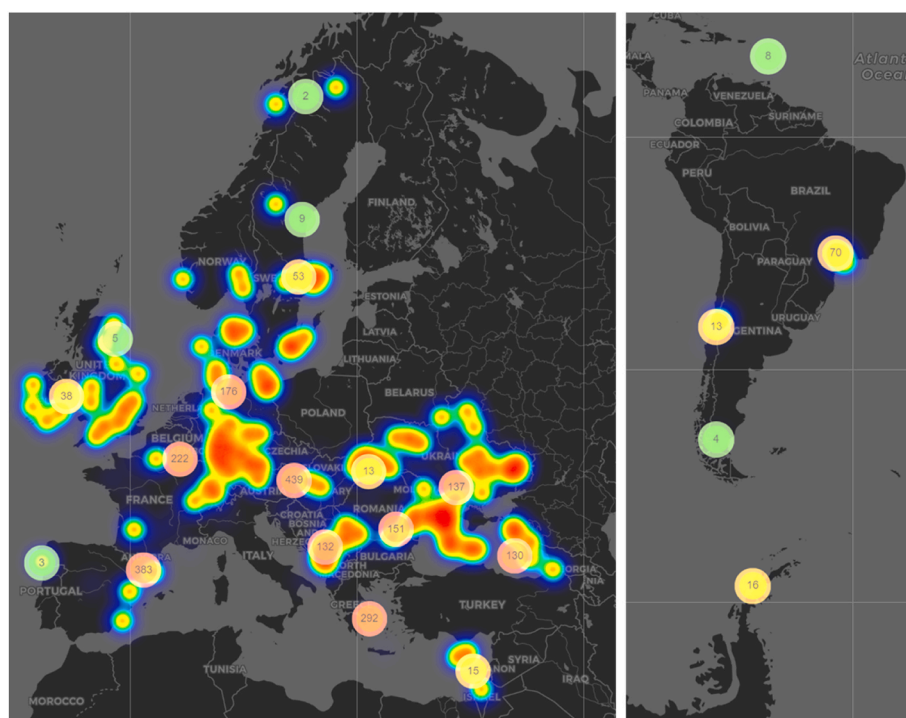


Fig. 4. Spatial distribution of digitally archived HRMS data in the digital sample freezing platform (DSFP) as of December 2021 for Europe, Latin America and Antarctica.

apex predators and other biota samples are available in many European institutions (ESBs, RCs and NHMs). By applying the presented integrated workflow for quality assured sample collection, processing, preparation and analysis, we demonstrate how the use of archived samples can be maximised for chemicals management, especially when apex predators are sampled in the same spatiotemporal context as potential prey species (Treu et al., 2022). Subsequent digital sample freezing of HRMS data allows for a fast provision of exposure data as well as for retrospective screening for chemicals that may be of interest/under assessment in future without the need of reanalysing the samples. To further enhance the connection between stakeholders of the DSFP (i.e. academia, regulators, industry) further guidance documents need to be developed on how to interpret and use data from apex species in chemical risk and hazard assessments. Currently, the expansion of digital collections in terms of spatial distribution, matrix type and time dimension remains a challenge. Key contributors to address these challenges are ESBs, RCs and NHMs. They have to be supported by policy as considered in the European PARC initiative (PARC, 2020). Such initiatives should also support collaborations among analytical laboratories, collections and field groups to ensure the flow of suitable samples for pan-European analyses. All these measures are expected to advance biomonitoring initiatives and ultimately increase the regulatory uptake of chemical data not only from apex predators but wildlife in general.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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