VDI

Federal Agency for Nature Conservation

DNA-based biodiversity analyses in nature conservation and environmental protection: What options do we have for standardisation?

A recommendation for action from research and practice

Florian Leese, Ljuba Woppowa, Miklós Bálint, Sebastian Höss, Henrik Krehenwinkel, Stefan Lötters, Kristian Meissner, Carsten Nowak, Philipp Rausch, Vera Rduch, Björn Rulik, Alexander M. Weigand, Jonas Zimmermann, Jan Koschorreck and Wiebke Zügh art

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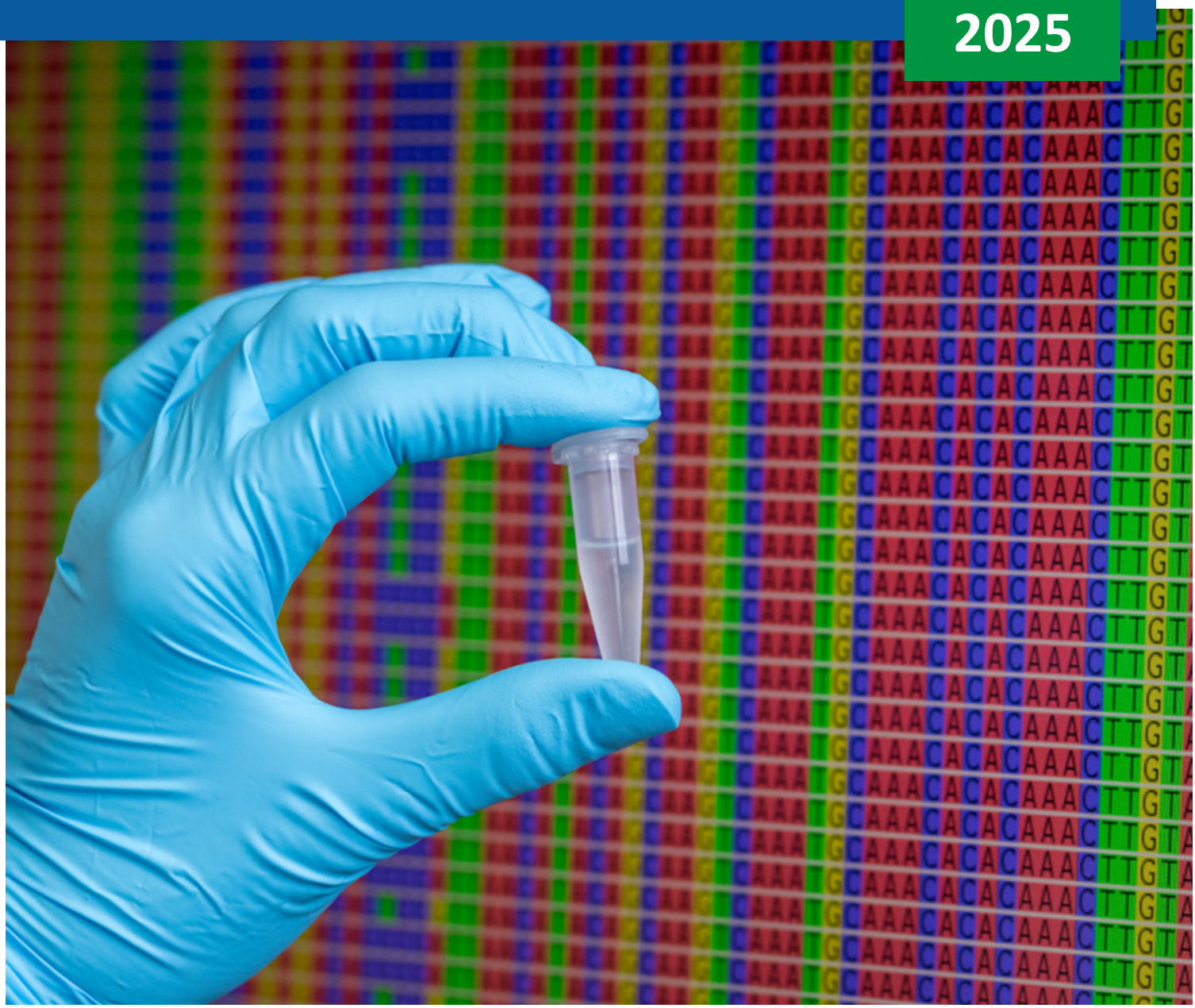

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Summary and recommendations for action

This recommendation for action analyses and evaluates the challenges and options for the standardised use of DNA metabarcoding in official nature conservation and environmental protection. Based on this, concrete solution options for standardisation and quality assurance as well as the necessary steps are presented. The recommendation for action summarises the results of the workshop "DNA-based biodiversity analyses in nature conservation and environmental protection: What options do we have for standardisation?", which was held by the Federal Agency for Nature Conservation (BfN) together with the Biodiversity Division of the VDI Society Technologies of Life Sciences (VDI-TLS) from 1-3 June 2022 in Schöntal Monastery (Fig. 1).

Fig. 1: Participants of the workshop "DNA-based biodiversity analyses in nature and environmental protection: What options do we have for standardisation?" in Schöntal Monastery. (© Woppowa, VDI)

The recommendations for action are aimed at political decision-makers in nature conservation and environmental protection, official environmental monitoring and experts in DNA metabarcoding and environmental and biodiversity monitoring.

Recommendations for action

- It is time to act now: Authorities are discussing DNA metabarcoding as a complementary method for biodiversity monitoring. For comparable and reproducible species lists, minimum technical standards as well as quality assurance from sampling to the compiled species list are needed.
- An essential prerequisite for the use of DNA metabarcoding in routine official operations is the regular exchange of designated experts for the planning and implementation of DNA-based monitoring programmes, laboratory testing and evaluation and reporting. This also includes clear terminology, standardisation at national (DIN or VDI) and international level (CEN or ISO) and close coordination with other countries. The latter is a prerequisite for the international recognition of the procedures and the comparability of the data.
- The established sampling routines of monitoring programmes must be adapted or extended to allow for DNA metabarcoding studies. The reliable assignment of species names requires quality-assured, public reference databases that are gradually expanded and made available by experts. In the medium term, the aim is to cover "dark taxa", i.e. groups of organisms that have hardly been studied so far.
- Minimum standards for the quality assurance of critical workflows and sample types are recommended for laboratory analyses. Quality assurance of DNA-based analyses requires measures on four levels:
    1. Framework conditions for the manner of sampling and laboratory operation, especially with regard to documentation and sample storage.
    2. Regular suitability tests of the involved laboratories by competent institutions, for example via ring tests.
    3. Minimum requirements of the laboratory tests through specifications for critical parameters such as number, size and preservation of samples, contamination-free working method, number of replicates, negative/positive controls and sequencing depth.
    4. Quality control of analysed samples *a posteriori*, e.g. via reserve samples or co-analysis of blind samples.
- It is necessary to institutionalise the coordination of quality assurance, for which there are experiences and examples of other measurement programmes, e.g. the Federal/State Measurement Programme (BLMP) of the coasts.

1 Background, motivation and goal

While DNA-based methods are already frequently used in research, their use in an official context is still rather the exception. For nature conservation and environmental protection authorities, the question of the maturity of DNA-based methods for biodiversity analyses and the associated standards as well as quality assurance measures is particularly important. These are crucial prerequisites for the inclusion of new methods in nature conservation and environmental protection monitoring programmes.

From 1 to 3 June 2022, the Federal Agency for Nature Conservation (BfN) together with the Biodiversity Division of the VDI Society Technologies of Life Sciences (VDI-TLS) organised a workshop on "DNA-based methods in nature conservation and environmental protection: What options do we have for standardisation?" at the Bildungshaus Kloster Schöntal.

The Schöntal workshop brought together experts from research and practice to derive concrete research and action steps for practice based on options for the standardisation of DNA-based methods. The focus was on DNA metabarcoding (hereafter referred to as "metabarcoding"), a method for compiling species lists based on bulk samples or environmental samples with DNA traces of organisms.

The Schöntal workshop was organised by a coordination team of the Federal Agency for Nature Conservation (BfN), the VDI Society Technologies of Life Sciences (VDI-TLS), the Federal Environment Agency (UBA), the University of Duisburg-Essen (UDE), the Botanic Garden and Botanical Museum / Freie Universität (FU) Berlin, Senckenberg Society for Nature Research Frankfurt (SGN), University of Trier (UT), Leibniz Institute for Biodiversity Change (LIB), University of Kiel, members of the VDI Advisory Board Biodiversity as well as experts from the environmental authority SYKE Finland and the National Museum of Natural History Luxembourg (MNHNL).

An empirical basis for the on-site discussions was provided by a survey on the standardisation of DNA-based methods, which was conducted prior to the workshop and included responses from experts from universities, research institutes, authorities, expert offices and contract laboratories. A total of 40 experts took part in the workshop and discussed the options and specific challenges of standardising metabarcoding with respect to four topics:

- Sampling
- Laboratory analysis
- Databases
- General quality assurance measures in official monitoring programmes.

This recommendation for action analyses and evaluates the options and challenges of the standardised use of metabarcoding in official nature conservation and environmental protection. Based on this, concrete solution options for standardisation and quality assurance as well as the necessary steps are presented. Finally, the importance of coordinated transnational processes for standardisation is highlighted and possible platforms for this are proposed.

2 Importance of biodiversity data in official nature conservation and environmental protection, application potential of DNA-based methods

Since the 1970s, environmental policy at national and European level has created the legal basis for the protection of nature and the environment and the conditions for scientific research to support their implementation. Long-term monitoring programmes make an important contribution to fulfilling the tasks of the federal government and the Länder. Biodiversity data make it possible to assess the state of ecosystems, to identify the reasons for adverse changes and to derive measures for the protection of nature and the environment. Biodiversity data are the basis for knowledge-based policy advice and provide information on whether measures taken are effective or whether there is a need for readjustment.

In Germany, the Federal Nature Conservation Act § 6 "Observation of nature and the countryside" regulates the tasks of the Federal Government and the individual states, such as the reporting obligations for the EU directives on the protection of habitats (HD), birds (BD), seas (MSFD) and invasive alien species (IAS). Further tasks of the federal government and the Länder result in particular from the comprehensive set of regulations of the EU Water Framework Directive (WFD) and the Washington Convention on International Trade in Endangered Species (CITES). Strategic guidelines for biodiversity research are the United Nations Convention on Biological Diversity (CBD), the National Strategy on Biological Diversity (NBS), the German Sustainable Development Strategy and the German Strategy for Adaptation to Climate Change. With the Zero Pollution Ambition and the EU Biodiversity Strategy 2030, the European Green Deal sets new impulses in environmental protection by aiming for ecosystems to be recovered, resilient and adequately protected by 2050. An important premise for success in all fields of action is comprehensive observational data for assessing the current state of and changes in biodiversity, as well as meaningful forecasts for the future. The importance of using new, innovative and efficient methods to complement the established classical methods is steadily increasing. DNA-based methods, along with remote sensing, acoustic data analysis and automated image recognition (AI), are among the promising methods for which there are already reliable experiences and practical examples.

Unlike research projects, which usually collect localised biodiversity and environmental data for a single year or a few years, government monitoring programmes are designed for temporal and spatial comparison and are often planned over decades. For meaningful assessments, all data must be comparable, reproducible, representative, valid and quality assured over the long term. New methods are only used once they have been sufficiently tested for routine operation, defined in procedural guidelines and backed up with quality assurance measures.

The use of DNA-based methods is already established in HD monitoring. Genetic species monitoring methods are used, for example, to record the conservation status of wolves, lynx and wildcats as well as various amphibians in water bodies. The nationwide insect monitoring currently under construction also offers great potential for the use of metabarcoding, e.g. for the analysis of bulk samples of flight-active insects (malaise traps, paint trays, etc.), ground-dwelling beetles and spiders (ground traps) or xylobiont beetles in the forest (cross window traps). As insect monitoring is a trend monitoring, not only evidence of the species composition, but also of the species' abundances is required, which is not yet possible with metabarcoding, at least not at present.

In addition, metabarcoding methods are being developed, e.g. for the detection of ingredients of protected plants in mixtures and highly processed products, in order to detect trade in illicit products (CITES agreement). In addition, projects are funded to explore the use of environmental DNA metabarcoding (eDNA metabarcoding) in WFD monitoring programmes (www.GeDNA.de) and for the structured collection of DNA-based water data in research databases that follow the FAIR "Guiding Principles for scientific data management" on data quality and availability, i.e. are findable (f), accessible (a), interoperable (i) and reusable (r). For the Federal Environmental Specimen Bank, experts from several research institutes are developing process descriptions for eDNA metabarcoding and other genetic methods. They make it possible to retrospectively examine archive samples from the last decades from oceans, inland waters and terrestrial habitats and thus close important gaps in our understanding of the development of biological diversity in ecosystems (www.TrendDNA.de). In addition, genetic methods will be included as new routine parameters in the investigation programme of the environmental sample bank. For future soil biodiversity monitoring, metabarcoding methods for recording soil biodiversity are being developed and tested.

These examples show that DNA-based methods for recording biodiversity offer a high potential for nature conservation and environmental protection. The prerequisites for their use in official monitoring are that they meet the requirements outlined above and provide robust, quality-assured and reproducible data. To ensure comparability, standardised procedures must be used that have proven themselves in practice. The standardisation of metabarcoding methods is an important step to ensure this.

Summary

- For official environmental monitoring, long-term data on biodiversity are of great importance for assessing the current status and changes.
- Data must be representative, comparable, reproducible and valid in the long term and be findable, accessible, interoperable and reusable according to FAIR principles.
- DNA-based methods for biodiversity surveys have hardly played a role in official monitoring so far, but they are all the more important in research.
- Standardisation is an important next step in opening up the scientific potential of the methods for official practice.

3 Importance of standards in monitoring programmes

Standards, technical rules, norms or guidelines are all designations of documents that are defined in Germany, Europe and worldwide by rule-making institutions within the framework of a clearly regulated, transparent and participatory process. The documents define the terminology of the process or product in question and formulate concrete requirements and recommendations for the processes and products. In this Recommendation for Action we use the term "standards". The process of setting rules is called "standardisation". The German Institute for Standardisation (DIN) publishes standards and is the largest national rule-setter. DIN has the sole state contract that allows it to represent Germany in terms of standardisation at the international standardisation bodies, CEN (Europe) and ISO (International). While CEN standards must be adopted as national standards in the national body of rules and regulations, the decision to adopt ISO standards lies with DIN. After DIN and the Association for Electrical, Electronic & Information Technologies (VDE), the VDI is the third largest rule-setter in Germany. The standards published by VDI and DIN are to be regarded as equivalent and often cover complementarily different areas. The legislator decides on their mandatory use.

In basic research and method development, standards are often perceived as extrinsic requirements that slow down innovation. This is one of several prejudices (Box 1), because standards are a prerequisite for the application of methods to obtain comparable results. They make new technologies suitable for general application. With regard to nature conservation goals, for example, the Bundesverband beruflicher Naturschutz e.V. (Federal Association for Professional Nature Conservation) formulates in this context: "Standards are modern instruments with which nature conservation goals can be achieved better, more economically and with greater acceptance". How successfully standards can be used is explained by certain criteria.

Technical rules as a tool for quality assurance

In principle, standards are the basis of quality assurance. This is particularly relevant and advantageous for research-related standardisation, because standards define evaluation benchmarks, such as the state of the art in science and technology, provide concrete assistance and thus facilitate the transition from research to practice, i.e. from invention to innovation.

For new technologies, standards increase acceptance and create confidence in the safety and quality of the product or process. Through the involvement of all interested parties, the development according to the consensus principle as well as the two-stage publication as a draft and, after a public objection procedure, as the final standard, transparency and quality assurance are guaranteed in the development process of the technical rules. A regular review every 5 years ensures that a standard is up-to-date and permanently available. National standards also often serve as national positions or basic documents for European and international regulation.

Minimum requirement

The possibility of technological development is taken into account by standardising so-called minimum requirements. Processes (procedures) and products (e.g. analytical equipment or sampling apparatus) are not specified down to the smallest details (and thus possibly inhibit developments), but it is specified which properties or characteristics the standardised process or product must at least fulfil, e.g. a metrological detection limit or a product lifetime. The way in which this minimum requirement is fulfilled is up to the user or developer. The definition of minimum requirements gives e.g. laboratories or device developers room for manoeuvre and the possibility to further develop their processes and devices. Minimum requirements must at least be met, but technological improvements are gladly used by the user - and may even lead to an update of the technical rule.

Metrology

The aim of data collection is a precise and correct measurement of certain parameters of the actual state (Fig. 2). This can be, for example, the measurement of body temperature in a hospital, the layer thickness of a circuit board in electrical engineering, or the number of species of insects in a meadow. The result should be independent of the laboratory carrying it out. Standards provide the basis for this. The technical setting of rules has a special significance for the comparability of measured values. Both in the case of recurring measurements by a laboratory and in the case of comparisons of results from different laboratories, standardised procedures are required for carrying out the measurement and, if necessary, for checking the results. Only in this way can it be prevented that false or inaccurate measured values are obtained in the most diverse sectors and contexts. In order to guarantee comparability of the measured values, special boundary conditions must be observed. Experts speak of comparison conditions and repeatability conditions.

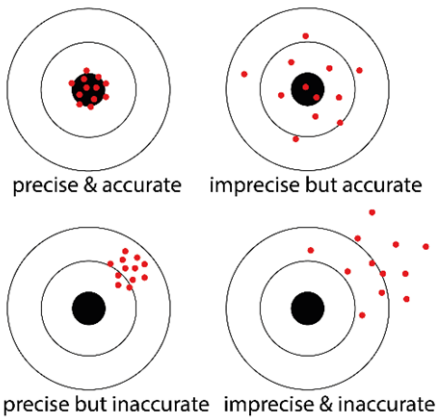

Fig. 2: Certainty (precision) and accuracy of a measurement. For a biodiversity survey, this means that the method can deliver the actual species composition (= black centre) with as little deviation as possible (= scattering of red dots) in repeated or independent measurements. (© Leese modified according to DIN ISO 5725-1 Accuracy (directness and precision) of measuring methods and results - Part 1: General principles and terminology (ISO 5725-1:1994))

Liability

The application of standards is basically voluntary. Standards are not regulations, but recommendations under private law. However, the process of setting technical rules implies a strong presumption that the application of the technical rule corresponds to the state of the art and leads to "correct" results. This presumption also often comes into play in judicial expert opinions. Nevertheless, the users are free to modify the standardised procedure for justified reasons, because an application case requires this.

However, the use of standards can be required within the framework of statutory environmental observation and monitoring. If the legislator "tightens up" the technical rules in its laws, i.e. makes them binding, the rule-making has a state-relieving effect. Important examples are e.g. TA Luft (Air monitoring) or WFD. Then the non-application of a standard means a violation of the law and can be punished.

Box 1: Standards – myths and facts

Misconception #1: Standardisation prevents progress

Fact: Technical rules must be reviewed every 5 years to ensure they are up to date. Standards often lead to a technology becoming suitable for general use.

Misconception #2: Standards are regulations, therefore binding to comply with

Fact: Standards are not regulations, but recommendations under private law. As soon as standards are prescribed by law, compliance with them is mandatory.

Misconception #3: DIN standards are binding, VDI guidelines only recommendatory

Fact: DIN standards, VDI guidelines and other rule formats are equivalent and their application is recommended. The binding nature of the technical rule is determined by the legislator.

Misconception #4: CEN and ISO standards are equivalent

Fact: CEN standards must be transposed into national standards throughout the EU. ISO standards can be transferred into national standards, the decision is made by the competent national mirror committee (see also Annex). The binding nature of the technical rule is determined by the legislator.

Misconception #5: Standardisation is intransparent

Fact: Standards are created according to defined and transparent processes (e.g. VDI 1000, DIN 820). Important principles are consensus principle, appeals procedure, involvement of all interested parties.

Misconception #6: Standards are public domain, you can copy them freely

Fact: DIN standards, VDI guidelines and many other standards are subject to a fee and are distributed by Beuth-Verlag Berlin. The costs are used to finance the work of the standards development department.

Further information: VDI blog - six misconceptions about guidelines and standards (www.vdi.de).

Since DNA-based monitoring methods are currently established mainly in research and hardly in official practice, there is now the possibility to start implementing quality assurance along the entire process chain from sampling to the transmitted result, i.e. the species or taxa list, with the help of the standardisation of procedures and the establishment of a standardised quality management.

Summary

- Standardisation is the formal process of setting rules and can take place at national (e.g. DIN, VDI) or international (CEN, ISO) level.
- Standards define parameters of a product or process that must be met in order to arrive at a correct, precise measurement, i.e. correct, comparable and reproducible species lists in biodiversity monitoring.
- Especially for new technologies, minimum standards increase acceptance and ensure quality-assured data.

4 DNA-based methods for recording biodiversity

DNA-based methods for temporal and spatial recording of biodiversity have developed rapidly over the last two decades. In particular, the technique of metabarcoding has great potential for the generation of qualitative species and taxa lists for official monitoring. Species and taxa lists can be generated from complex bulk samples (e.g. insect traps or net catches) but also directly from environmental samples such as water, soil or sediment and air based on environmental DNA (eDNA) (Box 2). DNA-based analyses can be technically scaled up. This means that a large number of samples, even many dew samples with tens of thousands of individuals, can be processed in days to weeks. Also, many organisms can be quickly identified to species level, for which an identification based on external characteristics would be difficult or impossible.

In addition, metabarcoding offers insights into community diversity metrics (α-, β-, γ-diversity), the genetic variation of individual species in a mixed sample and the interaction of species in a community (e.g. via metabarcoding of gut contents).

Box 2: Sample categories for DNA metabarcoding

Sample categories for metabarcoding are roughly divided into "bulk" samples (aggregate samples from biological communities, e.g. catches from Malaise traps, etc.) and environmental DNA samples (eDNA samples). There are numerous definitions for these broad categories, which overlap considerably, especially with regard to microorganisms. In this document, the authors define them as follows:

Bulk samples consist of organisms that have already been removed from their substrate and preserved (e.g. a macrozoobenthos sample taken for analyses according to the Water Framework Directive or a jar with insect bodies from a Malaise trap). The aim of standardised bulk sampling is to ensure that the DNA of all organisms is preserved for metabarcoding.

Environmental DNA samples consist of an environmental matrix containing DNA of the target taxa (e.g. soil, water and air filtrates, intestinal contents). The aim of standardisation is to ensure that the DNA traces from the environmental matrix are suitable for metabarcoding, where even small organisms from the substrate are preserved.

Metabarcoding is an established method in science for the determination of numerous organisms in a sample. The method is based on the fact that different species differ genetically. Sequencing, i.e. the "reading out" of a characteristic section of the organisms' genetic material, the so-called DNA barcode, can be used to determine the identity of an organism by comparing it with a reference database. In metabarcoding, this is done for numerous organisms simultaneously. The process chain consists of the following components (Fig. 3):

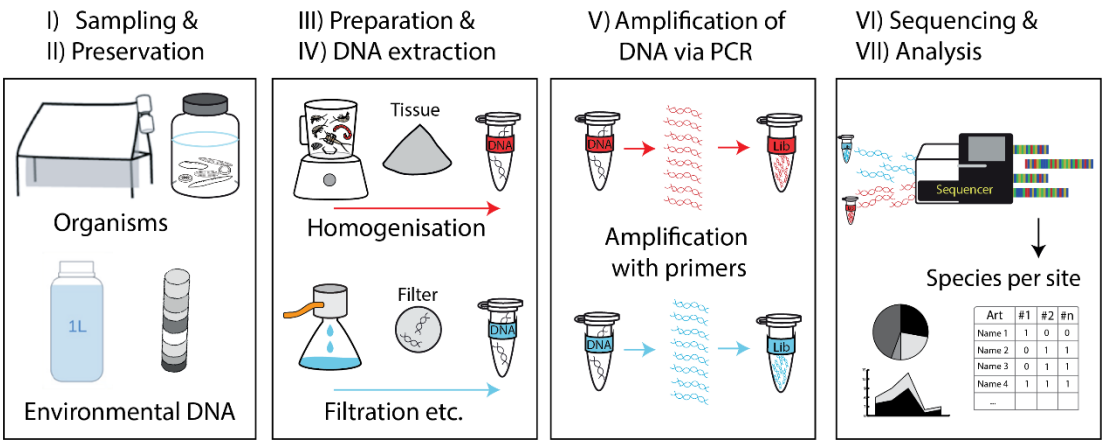

Fig. 3: Overview of the steps in DNA metabarcoding. (© Leese, University of Duisburg-Essen)

I) Sampling: The starting point of the analyses is the sampling of organisms in the environment. This can be done with specific traps or nets. Alternatively, a soil, sediment, water, air sample or other, e.g. plant/animal samples (see Tab. 1) can be used directly, from which the DNA is obtained without sorting (so-called eDNA) (Fig. 4).

II) Preservation: The sample material must be suitably preserved so that the DNA remains in good quality for laboratory testing. This can be done by drying, freezing or fixing in appropriate preservation or storage media.

III) Sample preparation: In the laboratory, the samples must be prepared for the analyses under pure (ideally target DNA-free) conditions. This means, for example, that the organisms must be size-fractionated and mechanically homogenised. In the case of eDNA samples, for example, the filter membranes must be crushed or sediment samples aliquoted.

IV) DNA extraction: The DNA must be isolated and purified from the samples using suitable methods. There are a number of different, already established procedures and process descriptions for this.

V) DNA amplification: DNA barcodes, which allow an unambiguous assignment of species, must be amplified enzymatically for all species present in the sample. This is done by the polymerase chain reaction (PCR). This amplification is necessary so that enough copies are available for subsequent high-throughput sequencing.

VI) Sequencing: The PCR-amplified DNA barcodes of the different organisms in a sample are read on a high-throughput sequencer. Up to several million sequences are generated for each sample in order to cover as many taxa as possible (small and large, common and rare).

VII) Evaluation: In order to arrive at species lists from the raw data (sequences from step VI), the sequences must be checked and filtered according to certain informatic criteria (signal vs. noise) and summarised according to similarity. The quality-checked sequences are then compared against a reference database. In this last step, species or taxa lists are created.

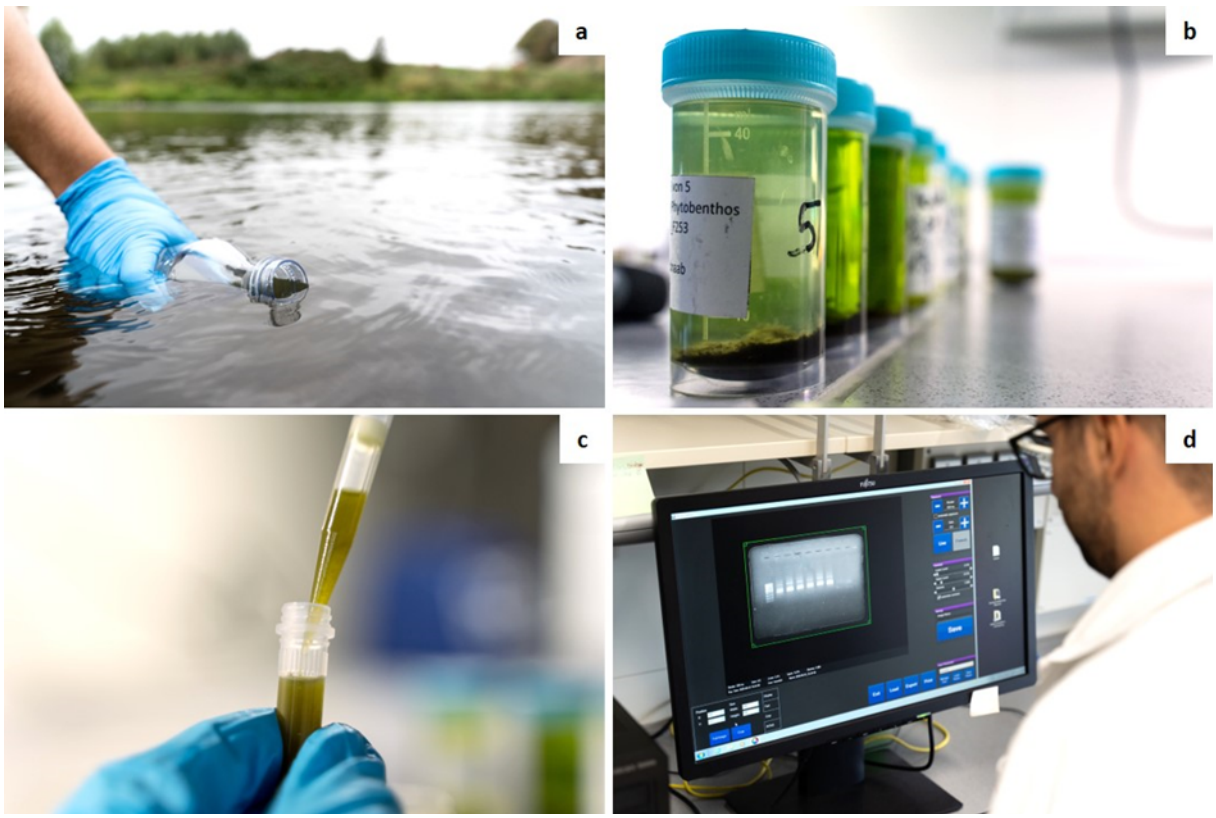

Fig. 4: Example images of environmental sampling and sample preparation for sequencing. a) Water sampling for subsequent environmental DNA analysis, b) Phytobenthos sample collected from stones using a toothbrush and stored in ethanol, c) Preparation of DNA isolation from a phytobenthos sample, d) Success control of a polymerase chain reaction using an agarose gel and UV light in the laboratory. (© Till-Hendrik Macher, GeDNA project)

Summary

- DNA metabarcoding refers to the analysis of species diversity in aggregate samples (e.g. from insect traps or net catches) or environmental samples (e.g. water, soil) by high-throughput sequencing of DNA barcodes from the sample.
- The metabarcoding procedure follows a clearly defined process chain from sample collection to species list, in which numerous parameters can be varied.

5 Sampling for genetic testing

Sampling must be well planned and documented, as the quality and size of the samples are the prerequisite for plausible and representative environmental monitoring data, and metadata are central to long-term sample archiving (e.g. in a museum or environmental sample bank). Errors in the design of the sampling scheme as well as during sampling are not revisable and are propagated to the reporting of results. Thorough selection of a suitable method and the required number of measurement points and measurement times are therefore crucial for the success of sampling and subsequent analyses.

An important building block for the use of genetic methods in official monitoring are standardised and validated sampling methods. In official monitoring, surveys in water, soil, air and biodiversity are already carried out according to standardised methods within the framework of classical monitoring (see standardised working instructions for insect monitoring or WFD ANNEX V).

The common sampling procedures in official monitoring are partly also suitable for DNA-based investigations, including sample preservation and storage (see Tab. 1). However, there is often still a need for research to identify suitable solutions. A central aspect is the stability of the DNA in the sample (Box 3). Even minor changes in a sampling protocol can influence DNA preservation and the suitability of the samples for metabarcoding, for example through different chemical preservation or sample storage.

The adaptation and standardisation of sampling procedures for metabarcoding shall ensure that 1) DNA obtained from a sample for metabarcoding is not degraded during temporary sample storage and 2) quantitatively and qualitatively similar DNA extracts are obtained from samples.

Box3: Sample quality and DNA metabarcoding

The suitability of a sample for metabarcoding is influenced by various factors:

DNA degradation: DNA can be degraded before or during sampling, preparation or sample preservation, which significantly reduces the DNA quantity and quality in a sample. Degradation occurs due to regular physico-chemical processes (hydrolysis, UV light). But biological processes are also relevant. DNA contains large amounts of phosphorus, an essential and often limiting nutrient for living organisms. Consequently, microorganisms are very efficient at using phosphorus from DNA samples if preservation does not inhibit microbial growth.

Contamination: All organisms possess DNA. Accordingly, contamination of samples by foreign DNA is easily possible during and after sampling if the samples come into contact with organisms from outside the sampling site or traces thereof (e.g. pollen or fungal spores, body parts, cell remnants) and if the sampling or processing is not clean. Contamination by exogenous DNA can be more or less relevant for certain sample types, depending on the general DNA concentration in a sample (samples with higher autochthonous DNA concentrations are more difficult to contaminate or the large dilution results in no false-positive detections) and the target species (contamination is lower when samples are processed in environments where no target species or their DNA are present).

Enzymatic inhibitors: Metabarcoding is based on an enzymatic amplification of DNA barcodes from the sample DNA (see Chapter 4). Samples often contain substances that limit the efficiency of the amplification enzyme. Different sample types and sampling methods are differently sensitive to analysis-inhibiting substances (so-called inhibitors).

Bulk samples of several target taxa can already be sampled by adapting existing international standards (ISO, CEN) for metabarcoding. International standards describe, for example, the sampling of the most important groups of invertebrate soil invertebrates (e.g. the standards DIN ISO 10381, DIN EN ISO 23611 of ISO/TC 190 Soil Properties, marine and freshwater macrofauna (e.g. DIN EN ISO 10870, DIN EN ISO 16665 of ISO TC 147 Water Properties) or benthic freshwater diatoms (DIN EN 13946 of CEN/TC 230 Water Analysis). There is also a CEN standard for the detection of allergenic pollen and fungal spores in air samples (DIN EN 16868 of CEN/TC 264 Air Quality). HELCOM protocols of the so-called Helsinki Commission describe how various biotic and abiotic samples of the Baltic Sea are to be taken (https://helcom.fi/action-areas/monitoring-and-assessment/monitoring-manual/). The focus of these standards is on morphology-based identifications so far. Several standards and widely used standard operating procedures are already focused on DNA endpoints. For example, there is an ISO standard for the extraction of microbial soil DNA that can be adapted for the collection of soil eDNA for other groups (DIN EN ISO 11063 of ISO/TC 190 Soil Properties). Similar standards apply to sampling standards for diatoms and macrozoobenthos. Furthermore, the European Centre for Soil Data has already gained experience with metabarcoding in the context of large-scale soil sampling in its pan-European Land Use and Coverage Area frame Survey (LUCAS) (https://esdac.jrc.ec.europa.eu/projects/lucas). The same applies to the monitoring of the Danube by the International Commission for the Protection of the Danube River (ICPDR). In the fourth international Joint Danube Survey, classical and DNA-based analyses were successfully carried out in parallel (https://www.danubesurvey.org/jds4/). In this context, the draft standard for aquatic eDNA sampling was published at the beginning of 2022 (DIN EN 17805). These standardised procedures explicitly take into account DNA-based taxonomic identification, but they still need to be extended, adapted or specified for concrete monitoring programmes.

Tab. 1: Suitability of current and future sample types for DNA metabarcoding and need for research. Expert rating. Suitability high: dark blue; medium: blue; low: light blue; Research needs high: brown; medium: orange; low: yellow. * eDNA.

	Sample type	Sampling		Sample preservation		Long-term storage	
		Suit-ability	Need for research	Suit-ability	Need for research	Suit-ability	Need for research
Today`s monitoring	Soil invertebrates	high	low	medium	medium	medium	high
	Soil microorganisms	high	low	high	low	high	low
	Macrozoobenthos, freshwater phytobenthos and plankton	high	low	high	medium	high	medium
	Benthic diatoms	high	low	high	low	high	low
	Fish / amphibians*	high	low	high	low	high	low
	Pollen	high	medium	high	high	high	medium
Future monitoring	Flying and epigeic insects	high	high	high	medium	low	high
	Pollen or plant traces on insects*	high	high	high	medium	high	medium
	Traces of insects on plants*	medium	high	medium	high	medium	high
	Pellets / droppings*	medium	medium	high	low	high	low
	Groundwater fauna, freshwater, meiofauna	high	high	high	medium	medium	high
	Water (total eDNA)	high	low	high	low	high	low
	Sediment (total eDNA)	high	low	high	low	high	low

Challenges

The large number of already selected and future sample types (see Tab. 1) from eDNA from soil to fungal spores from air is the biggest challenge for standardisation. Existing sampling strategies need to be considered for each sample type, even if for some no international or generally accepted standard procedures exist yet. Consequently, standardised sampling procedures need to be developed on a problem-specific basis in cooperation with metabarcoding, scientific and regulatory monitoring experts. In addition to the practical aspects of actual sampling and sample preservation, this development must also take into account aspects of experimental design (e.g. technical replication, spatial and temporal coverage).

A particular challenge with respect to sampling for DNA-based biodiversity monitoring is the long-term storage of samples or DNA extracts. Suitable long-term storage can be of great importance for quality assurance and future research. Current practice is heterogeneous and depends on official requirements and the specific availability of storage space and expertise. Whereas DNA extracts have a limited volume and can usually be stored for long periods frozen

or freeze-dried, the expected volumes of bulk samples from monitoring programmes, often with many large-volume vessels, pose a major logistical challenge for many institutions.

Important aspects for long-term storage are medium and temperature, distributed/fail-safe storage, curation, sample labelling and linkage to different databases (taxonomy, barcodes, publications). As long-term storage is labour and expense intensive, standardisation efforts must also take into account the often limited resources in terms of available funding and working time.

Documentation of metadata is a crucial aspect of sampling. Metadata describe various properties of metabarcoding samples, such as target organisms, sample volume, the area represented by a sample, collection method, capture medium, preservation method, sample container, date of sampling, coordinates, etc. The metadata are collected individually by the actors involved in the project. The metadata are collected individually by the actors involved in the project. However, the incorporation of metabarcoding as a method of regulatory monitoring requires a standardised collection, storage and sharing of metadata according to the FAIR data principles. This is essential for the inventory, comparison and reuse of samples and associated results. Metadata should be collected according to existing open source metadata standards for biodiversity research (https://tdwg.org/).

Research and communication needs

The variety of sample types and sampling strategies is particularly large for bulk samples (see Tab. 1). In order for metabarcoding to find a broad and quality-assured application and to lead to comparable results, minimum standards must be adapted or newly developed that take into account the special features of the individual sample types and sampling methods (see above). There are numerous aspects to be considered in standardisation for each type of bulk sample (e.g. mass/volume) required for DNA extraction (DNA extraction is generally performed from relatively limited sample volumes), size sorting, cleaning prior to homogenisation (e.g. removal of stones), division of the sample into technical replicates, and sample homogenisation. The preservation of the samples must also be determined for each sample type before processing in the laboratory; for example, with regard to the concentration and composition of the killing and preservation liquids or the need for subsequent freezing or drying.

eDNA samples generally have low target DNA concentrations compared to the total substrate volume and are therefore more susceptible to contamination with DNA from non-target taxa. The minimum standards for eDNA should therefore describe general strategies for contamination control during sampling, e.g. with regard to the inclusion of negative and positive controls during sampling, the use of contamination markers, and the preparation of recommendations for handling or avoiding cross-contamination. Here, the new CEN standard EN 17805 can be built upon.

In principle, an exchange between experts in sampling, laboratory testing and planning of monitoring programmes is essential so that the methods developed can be integrated into routine operations, i.e. are practical. Standardised terminology facilitates communication between experts with different backgrounds. This also applies to the downstream analyses. Examples for standardised terminology exists for biomonitoring of soil (ISO 11074) and water (ISO 6107).

Summary

- Sampling is a crucial step because errors here affect all subsequent steps in the process chain.
- Current sampling in official monitoring is partly already compatible for DNA analyses, but partly the sampling methods and strategies have to be adapted.
- All relevant sampling information must be recorded in a standardised manner, as it has an impact on the analysis.
- There is a need for research with regard to the designation of concrete work steps for the different sample types as well as with regard to the long-term storage of the samples.

6 Laboratory analysis DNA metabarcoding

After sampling, the generation of metabarcoding data comprises two basic steps: 1) the laboratory work and 2) the analysis of the generated sequence data. These two steps in turn comprise many individual steps, which must be well documented. For example, the laboratory work includes the isolation of the DNA from the sample, the amplification of the isolated DNA via PCR, the subsequent labelling of the individual samples with characteristic identification sequences (so-called indexes), and the subsequent high-throughput sequencing. Each of these steps can be extended by various intermediate steps and modifications of the reaction conditions in order to optimise the methodology, e.g. for a specific sample type (water vs. soil sample, malaise trap vs. macrozoobenthos mixing sample, etc.) or taxonomic group (vertebrates, insects, molluscs, etc.). Also, many different reagents are currently offered and used for the respective work steps. From a purely combinatorial point of view, this results in an extremely complex picture of countless process descriptions that are currently used for metabarcoding.

Many of the methods and steps used in metabarcoding procedures are very robust to variation, e.g. different DNA polymerases or primers produce comparable results (Fig. 5). Different metabarcoding methods can produce identical taxa lists as long as some basic steps are followed. Deviations are especially common for rare species. Existing guides provide a good overview of particularly sensitive steps (see e.g. https://doi.org/10.3897/ab.e68634, http://dx.doi.org/10.25607/OBP-1884, https://www.gedna.de/data/).

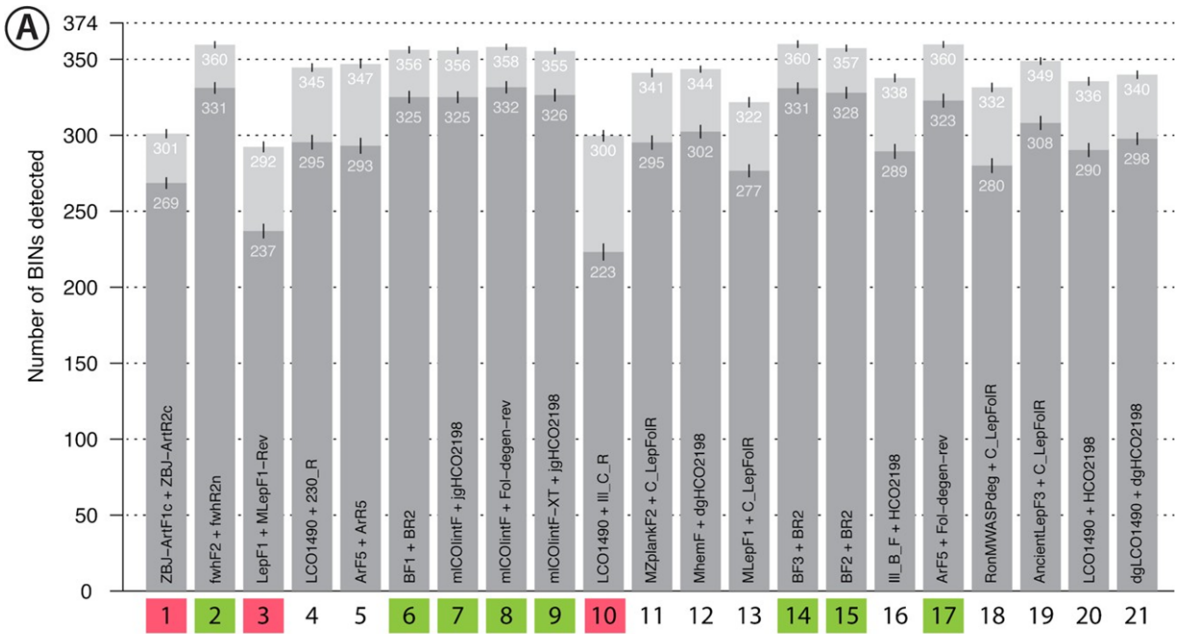

Fig. 5: Different primer combinations (X-axis) show different detections of the total of 374 possible target species, but many come to very similar results, which shows the robustness of metabarcoding method descriptions to variation. The primer combinations in green are particularly suitable, those in red particularly unsuitable. (Source: from Elbrecht et al. 2021; https://peerj.com/articles/7745/#fig-4)

Due to this basic robustness, strict standardisation of individual work steps is not necessary for many analyses. Overall, a flexible design of laboratory work and data analysis by different laboratories is acceptable. Exceptions are, for example, metabarcoding analyses of pollen from the air, because here even minor deviations in the methodology lead to major changes in the taxa lists. However, if an accurate taxa list can be generated with a method, a procedure description for official metabarcoding is to be considered suitable (result-oriented approach). Instead of prescribing exact and detailed process descriptions in metabarcoding, a check of the generated results for comparability, documentation of the work steps and use of suitable controls (reproducibility, measurement accuracy) should be considered the most important quality standard in metabarcoding (see Chapter 8).

Challenges and solutions

The main challenge is to produce comparable, correct and accurate species lists with metabarcoding in the long term. However, some basic steps are considered particularly important. The minimum requirements for metabarcoding process descriptions identified here mainly refer to the verifiability and plausibility of the generated metabarcoding data and not to details of individual work steps. These include in particular:

- The use of negative controls in all steps (DNA isolation, PCR amplification) is considered essential to identify contaminations in the workflow. Standardised handling, in particular how to deal with sequences in the negative controls and when a sample is to be discarded altogether, is required.
- For particularly sensitive analyses, such as pollen analyses or traces of pollen on insects or analyses of faeces and stomach contents, particularly clean working conditions must be ensured.
- Ensure sufficient sample-specific sequencing depth, especially for eDNA analyses and specimen-rich bulk samples.
- Technical and biological replication of individual steps can significantly improve the accuracy of the results. Rare taxa may only be detected with several replicates. Accordingly, concrete recommendations for the minimum technical or biological replication should be developed on a sample-specific basis.
- Particularly important is the precise documentation of work processes and results in accordance with the FAIR principles and recommendations on Good Laboratory Practice (GLP). For this purpose, project-specific formulations of minimum requirements for the publication of results and work steps used in the official metabarcoding are recommended.
- The storage of the samples used, or aliquots of them, in biobanks is strongly recommended to enable later verification.
- If necessary, sample-specific recommendations on the choice of suitable primer combinations or reagents to avoid inhibition of enzymatic reactions.
- The plausibility check of results by means of positive controls and/or "mock communities", i.e. artificial species communities with known taxonomic composition, is recommended. Such a mock community allows to test and validate the taxonomic accuracy of a procedure description (see Chapter 8).

- Interlaboratory comparisons are useful standardised quality assurance procedures to test the suitability of analytical laboratories without necessarily prescribing specific workflows. For example, only certified laboratories that correctly characterise an unknown mock community provided by a reference laboratory could be approved for metabarcoding analyses in official monitoring.

Need for research and implementation

There is a need for research on quantitative results of DNA-based biodiversity analyses. While metabarcoding already generates accurate qualitative taxa lists, further research in the future should focus on the generation of abundance or biomass information for taxa. There are methodological approaches here, e.g. using internal standards for calibration. Great potential is also seen in obtaining quantitative information by combining metabarcoding with other new monitoring methods, especially automatic image recognition for bulk samples. There is a great need for research and development at these interfaces. The reconstruction of biotic interactions using metabarcoding is also an important goal for future research. A comprehensive understanding of the complex interdependencies of different organisms in an ecosystem is essential for effective nature conservation and the use of DNA-based data.

There is less need for research than for implementation in the development of minimum requirements to guarantee quality-assured, comparable and verifiable results for official metabarcoding. This includes, for example, the development of uniform standards for the documentation and publication of results and workflows for official practice and research. Approaches to this exist, for example, in the context of NFDI4Biodiversity (Box 4). A future challenge is the development of diverse mock communities for different taxonomic groups that allow the accuracy of measurements to be verified. For groups that are rather species-poor in Germany, such as fish, this is relatively easy. For groups such as insects, with over 30,000 native species, on the other hand, the creation of a comprehensive mock community is difficult and would have to focus on a representative sample for quality assurance (see e.g. Fig. 5). Alternatively, or as a supplement, the establishment of interlaboratory tests for the suitability testing of species for official monitoring could be an important future task (see Chapter 8).

Summary

- The laboratory work and the analysis of the generated data are two essential steps in metabarcoding, which produces erroneous data if important aspects are not taken into account.
- Different metabarcoding protocols can deviate considerably from each other methodologically, but still generate accurate taxa lists. The great diversity of method descriptions used requires the introduction of minimum requirements for data quality assurance.
- The documentation of the work steps including the required metadata is crucial for the subsequent use of the data.

7 Database

Reference databases play a central role in DNA-based biodiversity analyses. Only the correct taxonomic assignment of a metabarcoding sequence from the collected sample to a DNA barcode in the reference database allows a reliable species determination. The quality of this species determination is thus indispensably dependent on the quality and scope of the DNA barcode reference databases. Depending on the organism group, different databases exist for different genetic markers. Science and authorities have different requirements for reference databases. Official biodiversity analyses are often subject to other questions and strict legal framework conditions (legally binding). In research, data with greater measurement uncertainty can also be used for biodiversity and trend analyses.

For the use of DNA barcode reference databases in the context of official monitoring, various prerequisites should be fulfilled as clear requirements (= standards). The reference databases should:

- enable the implementation of the FAIR principles for data,
- be taxonomically as well as nomenclatorically curated (constant quality assurance and control takes place),
- include raw data and appropriate metadata description, and reference supporting material where possible,
- ensure the transparency of the criteria for the creation of the entries deposited/provided in the database,
- contain citable entries (e.g. via DOI).

In addition to public databases, non-public, e.g. self-created databases are also used, especially in research. If these are used for species identification for monitoring purposes, they should at least be provided with a DOI and a short metadata description.

Challenge

A look at the databases used shows that they differ greatly in terms of organism groups, quality assurance, geographical coverage, completeness and available metadata (Tab. 2). While the data for vertebrates and the mitochondrial cytochrome C oxidase subunit 1 gene (COI) are very well recorded in BOLD (Barcode Of Life Data Systems) for European taxa, for example, they are insufficiently recorded for microalgae, plants and bacteria.

Tab. 2: Overview of particularly frequently used databases in DNA metabarcoding studies for assigning taxonomic names to sequences.

Database	Organism groups	Gene marker	Comment	Link
BOLD	Animals in particular	esp. COI	A large part of the sequences without morphological validation	http://www.boldsystems.org/
GBOL	Animals, plants, fungi and diatoms	e.g. COI, 18S, rbcL, ITS, trnIF/K, matK	Taxonomically very well curated by experts	https://gbol.bolgermany.de/
INSDC	All groups of organisms	All markers	A large part of the sequences without morphological validation	https://www.insdc.org/
Diat.barcode	Diatoms	esp. rbcL, partly 18S SSU	Taxonomically very well curated by experts	https://carrtel-collection.hub.inrae.fr/barcoding-database/diat.barcode
SILVA	Bacteria, Archaea and Eukarya	16S/18S, SSU and 23S/28S, LSU	Taxonomically well curated for the most part by experts	https://www.arb-silva.de/
PR2	In particular protists, focus marine	18S SSU	Taxonomically well curated for the most part by experts	https://pr2-database.org/
UNITE	Eukaryotes, esp. fungi	ITS	Taxonomic annotation by the expert community	https://unite.ut.ee/

Like the general structure of the reference databases, the information stored for individual reference barcodes, the so-called metadata, should also follow clear standards, such as those laid down by the Global Biodiversity Information Facility (https://www.gbif.org) (https://docs.gbif.org/publishing-dna-derived-data/1.0/en/). Minimum requirements for the data are defined as follows:

- Sampling location (documentation of sampling location: coordinates, habitat, etc.)
- Time or time window
- Collection / survey method
- Sample type
- Collector
- Identifier and time of identification
- Cultivator if necessary
- Laboratory analysis (in particular primers and, if applicable, also information on DNA extraction, PCR protocols as well as primary analysis data).

To achieve the best conditions for a reliable barcode reference database, additional information is important, in particular:

- Number of DNA barcodes per species and geographical coverage
- Expertise of identifier
- Barcodes from type material, life stage
- Accessibility of vouchers/receipts (vouchers must be available)
- Documentation (photo documentation) of the receipts.

This metadata can be used to determine the reliability of the assignment of a taxonomic name (in the best case, the species name) to a sequence or to reassign it if the taxonomy changes.

In addition to quality control when receiving reference barcodes and reference metadata, it is important to keep the databases up to date. This means constantly updating and completing information on taxonomy, ecology or endangerment. Synonyms should also be included and sequences adapted to new taxonomy. Thus, a constant harmonisation of morphological and molecular data and blending of synonyms is necessary. Federal taxa lists, operational taxa lists, and nomenclatural/taxonomic backgrounds should also be taken into account. The lists should be compatible with historical data and species lists. Since different databases are used, their compatibility and interoperability is an important aspect. In this way, the data basis for different analyses can be increased. Unfortunately, problems occur time and again when using and merging reference data from different databases. This is where the advantages of NFDI4Biodiversity (Box 4) lie, a project that aims to increase the interoperability of different databases (data models), because compatibility is feasible through data standards. Important international initiatives in this context are the GBIF and the Catalogue of Life (https://www.catalogueoflife.org). It is taken for granted that scientific names are used in databases.

In addition to the reference data, the public availability of the reference samples is also of great importance. Reference individuals can be stored in natural history collections, while molecular subsamples (DNA, eDNA, RNA, tissues, cells, etc.) are usually archived in biobanks. Specimen collections are not only indispensable when the underlying taxonomy of species changes, but also for the general verifiability and extensibility of results. Using museum organism samples or DNA samples frozen in biobanks, the coherence of new and old surveys can be ensured years later, e.g. when sequencing techniques have evolved (or new target genes are used), by extending the new analytical method to the archived samples.

Box 4: NFDI4Biodiversity

A central network with a view to making biodiversity data available is NFDI4Biodiversity, which acts as a consortium within the National Research Data Infrastructure (NFDI). It is currently scheduled to run for 10 years and is made up of around 100 partners, including scientific institutions, museums, natural history societies, state offices and other institutes and expert groups in the field of biodiversity and environmental data.

The cooperation is guided by the knowledge that actors from science, politics, nature conservation and landscape management need reliable data in order to be able to make better contributions to the conservation of biodiversity. The use of DNA-based data is also an important data source in the framework of NFDI4Biodiversity and guidelines for the handling, accessibility, analysis and networking of data are being developed in cooperation with de.NBI, ELIXIR and GBIF using TDWG standards (ABCD-DNA and DarwinCore).

NFDI4Biodiversity connects previously unconnected database infrastructures, not only of scientific institutions, but also of authorities, citizen scientist and expert groups, and harmonises workflows in a national and international context. In this way, further data repositories and tools are mobilised for collaborative use, which enables simplified handling for the user (e.g. linking of GBOL metabarcoding data with origin data from GBIF and Red List data). Furthermore, database tools are generated to identify duplicates, implement quality filters and make taxonomic changes in different lists traceable and transparent (e.g. changes in taxonomy in the Red Lists, GBIF and co.).

At the same time, NFDI4Biodiversity offers numerous workshops and trainings for the community and carries out important lobbying work for the safe and competent handling of data that is to be made available for broad and responsible use. Currently, there are 23 use-cases in NFDI, which are supposed to provide access to modern technologies and a comprehensive stock of biodiversity and environmental data through their work. In addition, there is a strong focus on the development of a cloud-based tool (both text-based and with a graphical user interface) so that users can, for example, upload metabarcoding data, quickly analyse it with the appropriate computing power and deposit it in the INSDCs (Tab. 2), thus enabling the link to the metadata.

More information: https://www.nfdi4biodiversity.org/

Need for research and continuation

In order to sustainably develop the full potential of DNA-based methods for official monitoring, high quality standards of database entries, open access to databases as well as alignment and harmonisation with international taxonomic frameworks are crucial. For Germany, an essential basis has been created with the German Barcode of Life Project (GBOL) (Box 5). Overall, however, long-term financing of the database infrastructure is necessary so that personnel and technical changes are guaranteed with regard to usability, even in the event of changes in nomenclatural or technical aspects. Maintenance also includes how databases are maintained and made available. As a general benchmark, it is important that databases are well secured, cloud-based and decentralised. Furthermore, routine filters should be implemented, e.g. to issue corresponding warnings in case of non-plausible entries (habitat, geography). An important scientific accompaniment to the data banks is the publication of data (annotated publication of barcodes).

Box 5: The Discovery of Unknown Diversity - GBOLIII "Dark Taxa"

The possibility of covering groups of organisms in databases depends primarily on two prerequisites: 1) the state of knowledge about this group of organisms (the species are known, named and described so that they can be identified), 2) the experts who can identify species of these groups. This is the only way to create the essential link between the identified specimen and the DNA barcode (with the associated data) in the reference database. If one of the prerequisites is not met, it becomes more difficult to include organisms in the reference databases. However, if both conditions are not met, it becomes virtually impossible to include organisms in the reference databases. These groups of organisms for which there is neither knowledge nor expertise are referred to as "dark taxa".

As an example, the Diptera (flies and mosquitoes) and Hymenoptera (bees, wasps and ants) are mentioned here, so-called megadiverse insect orders, which are represented in Germany alone with about 9,500 and 9,800 species, respectively. Of particular importance, both in terms of number of individuals and species, are mosquitoes, some groups of flies and the parasitoid Hymenoptera (i.e. species that develop on or in other insects). It is precisely these insect groups whose share of the total diversity actually found in environmental and bulk samples is enormous (often over 70% of the individuals found) - but which cannot be determined at species level. These dark taxa cannot currently be included in work in the field of biodiversity monitoring, nature conservation or ecology, let alone be evaluated for their role and usefulness in natural or man-made ecosystems and their potential danger. Even in Germany, only just under half of the approx.

33,000 insect species in Germany have been molecularly recorded. In addition to insects, there are dark taxa in many other groups of organisms (millipedes, spiders, mites, nematodes, protists, etc.).

Within the framework of GBOL III: Dark Taxa, the third phase of the GBOL initiative (https://bolgermany.de), funded by the BMBF, the focus is on research into selected dark taxa from the Diptera and Hymenoptera and their inclusion in the reference database. Further research initiatives are needed to scientifically document unknown species and make them available for biodiversity monitoring via reference databases.

Databases with unique sequences (so-called amplicon sequence variants, ASVs), as established in GBOL III: Dark Taxa, can help to deposit unique DNA barcodes without previously assigned species names and to include them in monitoring. Ideally, this sequence can later be linked to a species name, if this species could be described or correctly referenced.

In addition to the databases, another focus should be on improving the machine readability and interpretation of the data. The use of international standards (Biodiversity Information Standards, TDWG, https://doi.org/10.35035/doc-vf1a-nr22) is important here. Furthermore, data require correct and detailed metadata descriptions, which leads to FAIR data availability. In addition to the sustainable provision of data according to FAIR principles, efforts should also be made to close the gaps mentioned in taxonomic groups or regions and to merge databases. NFDI4Biodiversity is also available to support this.

Summary

- Reference databases are crucial for the correct taxonomic assignment of DNA metabarcoding sequences to species names; however, not all organism groups are fully represented.
- Metadata standards for DNA barcode references are necessary for quality assessment.
- Long-term funding of staff for curation as well as technical solutions for the interoperability of different databases are prerequisites for the sustainable use of data in official monitoring.
- DNA of all surveys should be made available in public collections to ensure extensibility and verifiability of all results.
- Reference databases should follow FAIR principles.

8 Quality assurance in the overall context

All nationwide biodiversity monitoring programmes are carried out systematically and use uniform or standardised methods that are described in guidelines or manuals. Depending on the responsibility, comprehensive quality assurance of the generated data is carried out by specialist societies, Land authorities, and/or BfN. Quality assurance also includes quality controls, e.g. by means of post-testing of reserve samples in individual programmes and federal states. Furthermore, training courses are offered, e.g. on the identification of certain groups of organisms. In some countries, proficiency tests according to standardised procedures (ISO 13528; Proficiency Testing) are/were used. For example, in Finland, personal proficiency tests are offered for traditional taxonomists, which only distinguish the respective person, but not the laboratory in which they work.

The aspects described in Chapters 5-7 show that guidelines for DNA-based methods already exist in some cases and that in many cases minimum technical requirements can be specified and implemented for official monitoring. However, in order to obtain valid biodiversity data for official monitoring, the establishment of technical minimum standards is only one component of a quality management system in the long term. Current metabarcoding studies show that different laboratories - even when complying with the minimum standards - can arrive at different species lists. This is particularly problematic for the detection of rare species. Accordingly, it is important that the implementation of further elements of quality assurance is planned in the context of official monitoring. The levels of quality assurance can be represented in a hierarchical model with different levels (Fig. 6).

LEVEL 1: The basic element here is a general quality management system in which general work processes are defined, preferably elements from ISO 9001, 17025 or the OECD guideline on Good Laboratory Practice (GLP). For DNA-based monitoring, the general and sometimes very comprehensive aspects of work organisation from ISO 9001 are usually less important than concrete measures to ensure high laboratory standards (but see level 3). This applies in particular to the documentation of all work steps, the follow-up of data and the regular validation of laboratory work steps. A conformity assessment by a certification body does not appear necessary at the present time, but should be assumed as part of the official monitoring.

LEVEL 2: If an analytical laboratory fulfils these basic requirements (Level 1), standardised procedures for testing the suitability of analytical laboratories offer a concrete possibility for identifying suitable laboratories. This can be done, for example, by means of interlaboratory comparisons prepared according to international standards (ISO/IEC 17043) and in which deviations in measurement accuracy (ISO 13528) are specified. For chemical, medical, and also biological tests, there are testing laboratories that can carry out certification according to the criteria to be fulfilled in the proficiency test via interlaboratory comparisons. One example is the German Reference Bureau for Interlaboratory Tests and Certified Reference Materials (https://drrr.de/). Within the framework of marine monitoring in Germany, as well as in some countries for monitoring according to the WFD, such interlaboratory comparisons for chemical and traditional biological detection are also regularly carried out, e.g. by the testing laboratory of the Finnish Environment Institute (SYKE). In the interlaboratory comparisons, reference materials are issued to the participating laboratories and the analysis results are evaluated centrally.

Only laboratories that have successfully analysed a certain proportion of samples correctly (e.g. defined as variation between technical replicates and total number of correct positives and false positives) are certified as analytical laboratories. This is already used in England to certify analytical laboratories for eDNA-based great crested newt monitoring. This certification is time-limited. A similar step is conceivable and necessary for metabarcoding analyses. Such standardised proficiency tests are all the more important as the development of DNA-based technologies for biodiversity monitoring is far from complete. Reference materials can be created using taxonomically unambiguous or monocultured organisms or artificially amplified tissue from plants or animals (e.g. museums, authorities), or the DNA of the target organisms to be tested can be artificially synthesised and made available.

LEVEL 3: Based on the "outer levels", a laboratory is in principle qualified to perform metabarcoding analyses according to defined minimum requirements. These requirements are to be defined on a case-by-case basis and relate, for example, to the number of biological and technical replicates, negative controls used and internal positive controls to detect possible cross-contamination, sequencing depth, reference databases and FAIR principles, as well as stricter specifications for working methods for sensitive sample types (e.g. pollen analyses).

LEVEL 4: As a quasi final step in the quality assurance of a laboratory, downstream quality control is recommended for regulatory monitoring, e.g. via the analysis of reference samples, i.e. samples that receive a tissue or DNA combination known from the reference laboratory (see Level 2). These samples must be co-analysed by the contractor and the results provided together with the analytical results. The deviation of the results of these blank samples (= positive controls) can be used to quantify the reliability of the measurement of the real samples, and if the percentage of correctly assigned species falls below a defined minimum limit, the results are rejected as unreliable. With regard to the institutional organisation, the quality controls are carried out by certified laboratories, similar to Level 2.

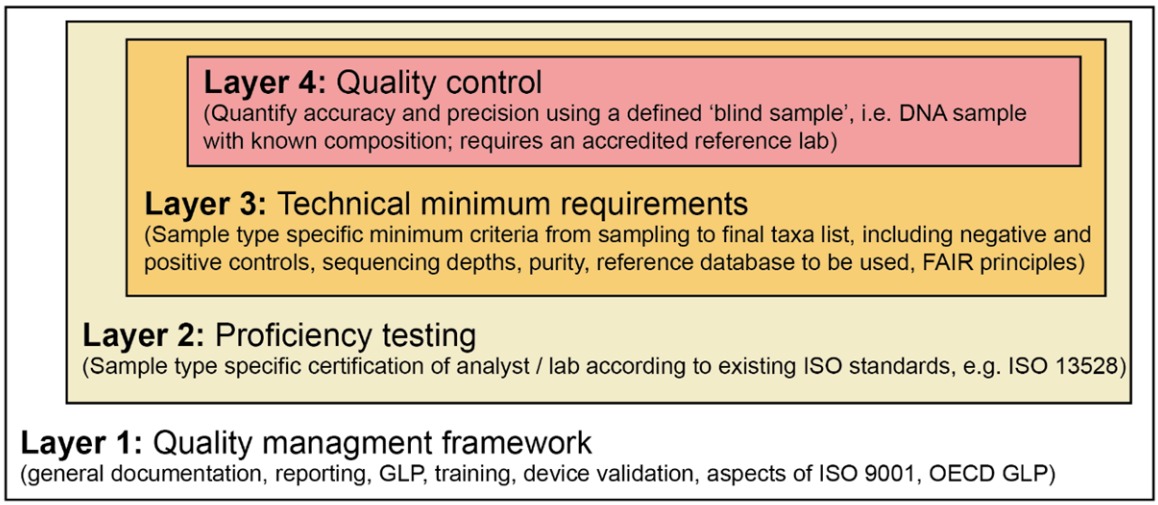

Fig. 6: Proposed levels for possible standardisation of DNA-based biodiversity analyses in official nature conservation and environmental protection. (© Leese, University of Duisburg-Essen)

Need for implementation

For regulatory monitoring, it is important to include a concrete list of successful criteria for the four different levels of quality assurance in the legal or sub-legal regulations. These must be partly specific according to the different sample types and monitoring programmes (especially level 2 and 3). In view of the implementation of European regulations for monitoring and cross-border investigations, it is advantageous if internationally accredited institutions carry out the quality assurance of the individual laboratories. Metrological institutions concerned with the traceability of measured values, their accuracy and their dissemination should support the standardisation of metabarcoding and other molecular methods for environmental monitoring at national and European level and include them in the test programmes.

Summary

- The use of minimum technical requirements is important for quality assurance, but does not guarantee high-quality data.
- Hierarchical quality assurance management is recommended for the official monitoring system.
- In particular, it is recommended to

i) establish a general conformity assessment of analytical laboratories (laboratory procedures, documentation, GLP),

ii) carry out an application-related suitability test (ring tests),

iii) define sample-specific minimum standards (replication, negative controls, purity, sequencing depth, reference databases),

iv) implement control samples for sampling as well as reference materials for quality control of laboratory results.

- Current test schemes according to international standards can be used for suitability testing and quality control.

9 Outlook for implementation

It is likely that many authorities will not carry out the genetic tests themselves, but will contract the service out to contract laboratories. In both cases, the laboratories involved must regularly prove that they are capable of carrying out the analyses in sufficient quality. To this end, quality assurance measures are possible at various levels; from the basic requirements for quality management in the laboratory operation, to checks on the quality of the measurement performance by third parties, to internal laboratory or project-related quality assurance measures (see Chapter 8).

Depending on the monitoring programme, authorities are likely to either commission the entire process of DNA-based assessment, from sampling and measurement to data analysis and evaluation, or contract out metabarcoding as a separate service that can be performed by DNA laboratories without expertise in sampling and assessment. Regardless of this, it is necessary that quality assurance measures begin at the sampling stage and accompany the entire process up to the analysis result. In order to lead the discussions into practice, it is conceivable as a first step to develop a quality assurance system on the basis of an example and to gather initial experience with it. Suitable for this would be eDNA metabarcoding for the assessment of fish diversity in water bodies, as there are already many concrete methodological guidelines, it is a manageable species group and there is a wide range of comparative data.

It is important that the quality assurance of metabarcoding studies is institutionalised in the official environmental assessment. For example, the Federal Environment Agency has already implemented a quality assurance unit for the morphological-taxonomic data in the Federal / State Coastal Monitoring Programme (BLMP). For the further internal process, a forum for transdisciplinary exchange on the topic of DNA-based methods is urgently needed at national, DACH or European/international level and should take place regularly, e.g. every two years, in dialogue between authorities and research institutions, but also with commercial providers.

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Fig. 6: Proposed levels for possible standardisation of DNA-based biodiversity analyses in official nature conservation and environmental protection. (© Leese, University of Duisburg-Essen) 32

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List of abbreviations

Abbreviation	Explanation
16S/18S and 23S/28S	Different ribosomal gene markers used in DNA-based biodiversity analyses to determine the different taxa
ASV	Amplicon sequence variants
BD	Birds Directive
BfN	Federal Agency for Nature Conservation
BLMP	Federal / State Coastal Measurement Programme
CBD	Convention on Biological Diversity
CEN	European Committee for Standardisation
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
COI	Gene marker; cytochrome C oxidase subunit 1 gene
DACH region	D (Germany), A (Austria), CH (Switzerland)
DAS	German Strategy for Adaptation to Climate Change
DIN	German Institute for Standardisation
DNA	Deoxyribonucleic acid
DNS	Germany's Sustainable Development Strategy (Deutsche Nachhaltigkeitsstrategie)
eDNA	Environmental DNA
EU	European Union
FAIR	Data quality and availability objectives: findable, accessible, interoperable, reusable
GBOL	German Barcode of Life
GLP	Good laboratory practice (guideline, recommendations, usually reference to OECD guideline on good laboratory practice)
HD	Habitats Directive
HELCOM	Helsinki Commission, international organisation for the protection of the marine environment of the Baltic Sea
IAS	Invasive alien species
ISO	International Organisation for Standardisation

Abbreviation	Explanation
ITS	Gene marker; internal transcribed spacer
LSU	Large subunit of the rRNA
LUCAS	European Land Use and Coverage Area frame Survey
matK	Maturase K, gene marker for the determination of plant taxa in particular
MSFD	Marine Strategy Framework Directive
NBS	National Biodiversity Strategy
NFDI	National Research Data Infrastructure
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase chain reaction
rbcL	Gene marker, ribulose-bisphosphate carboxylase
RNA	Ribonucleic acid
SSU	Small subunit of rRNA
TA Luft	Technical Instructions on Air Quality Control
TC	Technical Committee
trnL-F/K	tRNA gene markers for the determination of plant taxa in particular
VDE	Association for Electrical, Electronic & Information Technologies e.V.
VDI	Association of German Engineers e. V.
WFD	Water Framework Directive

A Annex: European and International Standardisation Activities Related to DNA Metabarcoding

A.1 European standardisation activities

A.1.1 CEN/TC 230 Water analysis (created 1989)

https://standards.cencenelec.eu/dyn/www/f?p=205%3A7%3A0%3A%3A%3A%3AFSP_ORG_ID%3A6211&cs=1F56318D14ADC18191F68173E68B16469

Secretariat: DIN (Germany)

Scope: Standardization in the area of water analysis including: - definition of terms; - sampling of water; - measurement; - reporting. Excluded are the limits of acceptability for water quality.

Structure: 10 working groups

Standards published: 225 documents

Work programme: 24 documents

A.1.2 CEN TC 230 / WG 28 Water Analyses DNA- and eDNA Methods:

https://standards.cencenelec.eu/dyn/www/f?p=205:7:0::::FSP_ORG_ID:2601978&cs=1D38954EB9D1DAF2DC920BEB143FCDE34

Secretariat: SFS (Finland)

Published Standards:

- CEN/TR 17244:2018 (WI=00230348)
  Water quality - Technical report for the management of diatom barcodes
- CEN/TR 17245:2018 (WI=00230349)
  Water quality - Technical report for the routine sampling of benthic diatoms from rivers and lakes adapted for metabarcoding analyses
- EN 17805:2023 (WI=00230395)
  Water quality - Sampling, capture and preservation of environmental DNA from water

A.2 International standardisation activities

A.2.1 ISO/TC 147 Water quality (created 1971)

https://www.iso.org/committee/52834.html

Secretariat: DIN (Germany)

Scope: Standardization in the field of water quality, including definition of terms, sampling of waters, measurement and reporting of water characteristics.

Structure: 6 Subcommittees

Standards published: 327 documents

Work programme: 42 documents

A.2.2 ISO/TC 190 Soil quality (created 1985)

https://www.iso.org/committee/54328.html

Secretariat: DIN (Germany)

Scope: Standardization in the field of soil quality: Soils in situ; Soil materials intended for reuse in or on soils, including dredged sub-aquatic soil materials (= excavated sediments).

Structure: 3 Subcommittees

Standards published: 179 documents

Work programme: 25 documents

A.2.3 ISO TC 331 Biodiversity (created 2020)

https://www.iso.org/committee/8030847.html

Secretariat: AFNOR (France)

Scope: Standardization in the field of Biodiversity to develop principles, framework, requirements, guidance and supporting tools in a holistic and global approach for all organizations, to enhance their contribution to Sustainable Development.

TC 331 Biodiversity will work closely with related committees (e.g. ISO/TC 190 Soil quality, ISO/TC 147 Water quality, ISO/TC 276 Biotechnology, ISO/TC 34 Food products) in order to identify standardization needs and gaps, and collaborate with other organizations to avoid duplications and overlapping standardization activities.

Structure: 5 working groups

Standards published: --

Work programme: 4

A.3 National mirror committees of DIN to European and international bodies

A.3.1 NA 119 DIN Standards Committee on Water (NAW)

European committees of NA 119

The Standards Committee accompanies the following European bodies at national level. For the committees marked accordingly, the secretariat is also at DIN.

https://www.din.de/de/mitwirken/normenausschuesse/naw/europaeische-gremien

International committees of NA 119

The Standards Committee accompanies the following international bodies at national level. DIN also provides the secretariat for the committees marked accordingly.

https://www.din.de/de/mitwirken/normenausschuesse/naw/internationale-gremien

NA 119-01-03 AA Water testing (mirror committee to CEN/TC 230, ISO/TC 147)

https://www.din.de/de/mitwirken/normenausschuesse/naw/nationale-gremien/wdc-grem:din21:54752592

A.3.2 NA 172 DIN Standards Committee on the Fundamentals of Environmental Protection (NAGUS)

International standardisation activities for the protection of biodiversity and ecosystems (din.de)

The DIN Standards Committee on the Fundamentals of Environmental Protection (NAGUS) is the national working body for interdisciplinary basic standardisation in the field of environmental protection at national, European and international level. NAGUS develops standards and specifications in the field of environmental management systems and environmental management tools.

NA 172-00-17 AA Biodiversity (mirror committee to ISO/TC 331)

The working committee NA 172-00-17 AA "Biodiversity" organises the German mirror work on ISO/TC 331 "Biodiversity" (secretariat: AFNOR, France) and, if necessary, further overarching standardisation work on this topic and ensures German participation in the work of the corresponding European and international standardisation (mirror work).

https://www.din.de/de/mitwirken/normenausschuesse/nagus/nationale-gremien/wdc-grem:din21:333002185

B Appendix: Conference programme

Bundesamt für Naturschutz

VDI

DNA-basierte Biodiversitätsanalysen im Natur- und Umweltschutz: Welche Optionen haben wir für eine Standardisierung?

01. bis 03. Juni 2022
Kloster Schöntal

Background information:

The scientific development of methods for DNA-based biodiversity analyses is progressing rapidly and their application is also of great interest for official nature conservation and environmental protection. In particular, official use, e.g. in the context of monitoring programmes, requires robust and standardised methods that provide reliable and comparable data.

The focus of the event is on "DNA metabarcoding". An introductory overview will be given of the current state of method development and its areas of application. On the basis of concrete projects, experiences with the use of DNA metabarcoding will be exchanged from the perspective of different actors. Subsequently, the participants will have the opportunity to discuss the following topics in four workshops and identify a possible need for standardisation:

I: Sampling and matrix (water, soil, air, land) II:

II: Laboratory work and data analysis

III: Reference databases and infrastructure

IV: Quality assurance for DNA-based monitoring

The identified challenges as well as the elaborated approaches for standardisation will be summarised, systematised, prioritised and published in a position paper by the participants and other experts after the event.

Circle of participants:

Representatives of research institutions, authorities, associations, expert offices.

Organiser:

Federal Agency for Nature Conservation (BfN) together with VDI Society Technologies of Life Sciences (VDI-TLS).

Venue:

Bildungshaus Schöntal Monastery, Klosterstr. 6, 74214 Schöntal;
Tel: 07943 / 8940; E-Mail: bildungshaus@kloster-schoental.de;
https://www.kloster-schoental.de

Conception and chairing of the conference:

Miklos Bálint, Senckenberg Research Institute and Nature Museum Frankfurt; Sebastian Höss, ECOSSA; Jan Koschorreck, Federal Environment Agency; Henrik Krehenwinkel, University of Trier; Florian Leese, University of Duisburg-Essen; Stefan Lötters, University of Trier; Carsten Nowak, Senckenberg Research Institute and Natural History Museum Frankfurt; Vera Rduch, Leibniz Institute for the Analysis of Biodiversity Change - Museum Koenig, Bonn; Christoph Scherber, Leibniz Institute for the Analysis of Biodiversity Change - Museum Koenig, Bonn; Ljuba Woppowa, VDI Society Technologies of Life Sciences (VDI-TLS); Wiebke Züghart, Federal Agency for Nature Conservation.

Costs:

Accommodation in single room incl. breakfast per pers./day: € 75

Payment is possible by EC card or Master/Visa credit cards.

Please register for the event by **30.04.2022** using the registration link:

Please also use the following link by **30.04.2022** for an initial allocation to the offered workshops I to IV (the final allocation will be made on site):

COVID 19 instructions and hygiene measures:

The respective rules of the Corona Ordinance Baden-Württemberg and the hygiene and protection concepts of the Bildungshaus Kloster Schöntal apply:
https://www.kloster-schoental.de

Arrival:

See Schöntal Monastery homepage:
https://www.kloster-schoental.de/meta/anreise.html

Programme

01. June 2022

Individual arrival

18.00-19.30	*Dinner together*
20.00	*Welcome, round of introductions, outlook on the next days, overview of the goals of the four parallel workshops*

02. June 2022

07.30-08:30	*Breakfast*

I Topic block: Plenary lectures

08:30	*Welcome*
08.40	*DNA metabarcoding in official nature conservation and environmental protection - status and perspectives Wiebke Züghart, BfN Bonn; Jan Koschorreck, UBA Berlin*
09.00	*Methods and development: How can we record biodiversity changes? What do we need from research (FINKA)? Christoph Scherber, ZFMK Bonn*
09:20	*Opportunities and risks of (still non-standardised) DNA-based methods for comprehensive biodiversity monitoring. Florian Leese, University of Duisburg-Essen, Essen*
09.40	***Coffee break***

II Topic block: Practical examples

10.10 — *Experiences from other fields working with genetic material, example wildlife genetics, solution: monopolisation*
Dr. Carsten Nowak, Senckenberg Research Institute and Natural History Museum Frankfurt

10.30 — *Soil: metagenomic surveys, standardisation and technological development*
Prof. Miklós Bálint, Senckenberg Research Institute and Natural History Museum Frankfurt, Biodiversity, Climate Research Centre

10.50 — *Insect monitoring,*
1. Case studies from the LTER-D project / Bavarian National Park Forest, Johannes Uhler Bavarian Forest National Park, Grafenau
2. Case studies from the Krefeld Entomological Society, Thomas Hörren, Krefeld Entomological Society

11.20 — *User/customer perspective: AIM Advanced Identification Methods, experience and needs*
Kirsten Morinière, AIM; Leipzig; Michael Traugott, SINSOMA, Innsbruck, Austria

11.50 — *Discussion*

12:15 — *Overview of four parallel afternoon workshops and division into working groups*

12.30-13.30 — ***Lunch***

13.30-14.30 — *Supporting programme: Guided tour of Schöntal Monastery*

III Workshops: How do we arrive at reliable and comparable species lists?

15.00	*Dividing into working groups, going to the workshop rooms*
15.15-18.30	*Workshops 1 to 4*
16:15	*Coffee Break*

Workshop 1 Sampling and media (water, soil, air, land)

Moderation	*Miklós Bálint; Carsten Nowak; Senckenberg Research Institute and Natural History Museum Frankfurt*
Objective:	*Summary of sample types for all four media currently collected and future needs for sample types.*
Content:	*We would like to discuss the following points:* *1. What is the diversity of scientific and regulatory sampling for water, soil, air, land?* *2. Is it possible to standardise sampling for certain media, e.g. analogous to the WFD? Malaise traps? What can be standardised? Summary of existing standardised sampling methods.* *3. Comparison of overlaps with classical methods* *4. Evaluation of whether the samples taken with classical methods are suitable for metabarcoding.*

Workshop 2 Lab work and data analysis

Moderation: *Henrik Krehenwinkel, University of Trier;*
Philipp Rausch, Kiel University, Institute of Clinical Molecular Biology (IKBM), Kiel

Objective: *The aim of the workshop is to identify minimum standards for the molecular and bioinformatic processing of metabarcoding data. In addition, particularly critical work steps that have a significant impact on the detected diversity will be identified.*

Content: *We would like to discuss the following points:*

1. Which analysis steps are not very sensitive and can be standardised? Which ones are very specific for certain questions and types of programmes or are particularly prone to error?

2. Can minimum standards for laboratory work and data processing be identified that a r e valid across different sample types and questions?

3. Can/should analytical steps also be carried out in official laboratories?

Workshop 3 Reference database and infrastructure

Moderation: *Vera Rduch, LIB / Museum Koenig, Bonn; Jonas Zimmermann, FU Berlin / BGBM*

Objective: *The aim of the workshop is to get an overview of the different reference databases with regard to their curation/data standards and to identify quality requirements as well as necessary performance requirements in order to ensure the basis for DNA-based biodiversity analyses in the context of official monitoring.*

Content: *We would like to discuss the following points:*

1. Which database is suitable for which group of organisms? What standards are the databases based on? Do data/adata standards exist?

2. What are the requirements for the documentation of genetic biodiversity analyses? What taxonomic resolution is aimed for?

(order/family/genus/species/population)? What are the quality assurance requirements for taxa lists?

3. What are the requirements for reference databases for official monitoring (cf. development of Red Lists, Federal Tax List)? How often would updates or adaptations be needed?

4. What about the compatibility of different databases?

5. What role does NFDI4Biodiversity play and what potential does it offer?

Workshop 4 Quality assurance for DNA-based monitoring

Moderation: *Florian Leese, University of Duisburg-Essen; Jan Koschorreck, Umweltbundesamt, Berlin; Kristian Meissner, Finish Environment Institute (SYKE)*

Objective: *The aim of the workshop is to discuss concrete quality assurance options for Identify DNA-based biodiversity analyses in the context of official monitoring and evaluate them with regard to their feasibility.*

Content: *We would like to discuss the following points:*

1. What are the assessment principles of different official environmental / bio(diversity) monitoring programmes? What data are available (species/taxalists/population sizes etc.) and what are they used for?

2. What are quality assurance procedures for existing surveys (ring tests, expert evaluation of reserve samples, photodocumentation, certification, proficiency tests, blind samples, etc.)?

3. What are the minimum requirements for quality assurance of existing monitoring programmes?

4. Which quality assurance measures are directly suitable for DNA-based surveys? Where are further, standardised procedures needed and where can one hope for or trust in "self-regulation"?

18.30 *Dinner*

19.30 *Summary 1st day, cosy get-together*

03. June 2022

07.30-08.30	*Breakfast*
08.30	*Standardisation: Quality and innovation are not a contradiction Florian Leese, Kristian Meissner, Ljuba Woppowa, Jonas Zimmermann;*
08.50	*Going to the workshop rooms*
09.00	*Continuation of the four parallel workshops*
10:30	*Coffee break*
11.00	*Presentation of the workshop results*
12.00	*Summary, next steps, conclusion and closing*
12.30-13.30	***Lunch together***

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“BfN Schriften” are a series of publications published non-periodically since 1998 by the editorial team of the Federal Agency for Nature Conservation (BfN) in Bonn. They can be produced at short notice and contain, among other types of publication, final reports of research projects, workshop and conference reports, working papers, and bibliographies. Many of the “BfN Schriften” are available digitally. Printed editions can also be produced in small print runs.

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