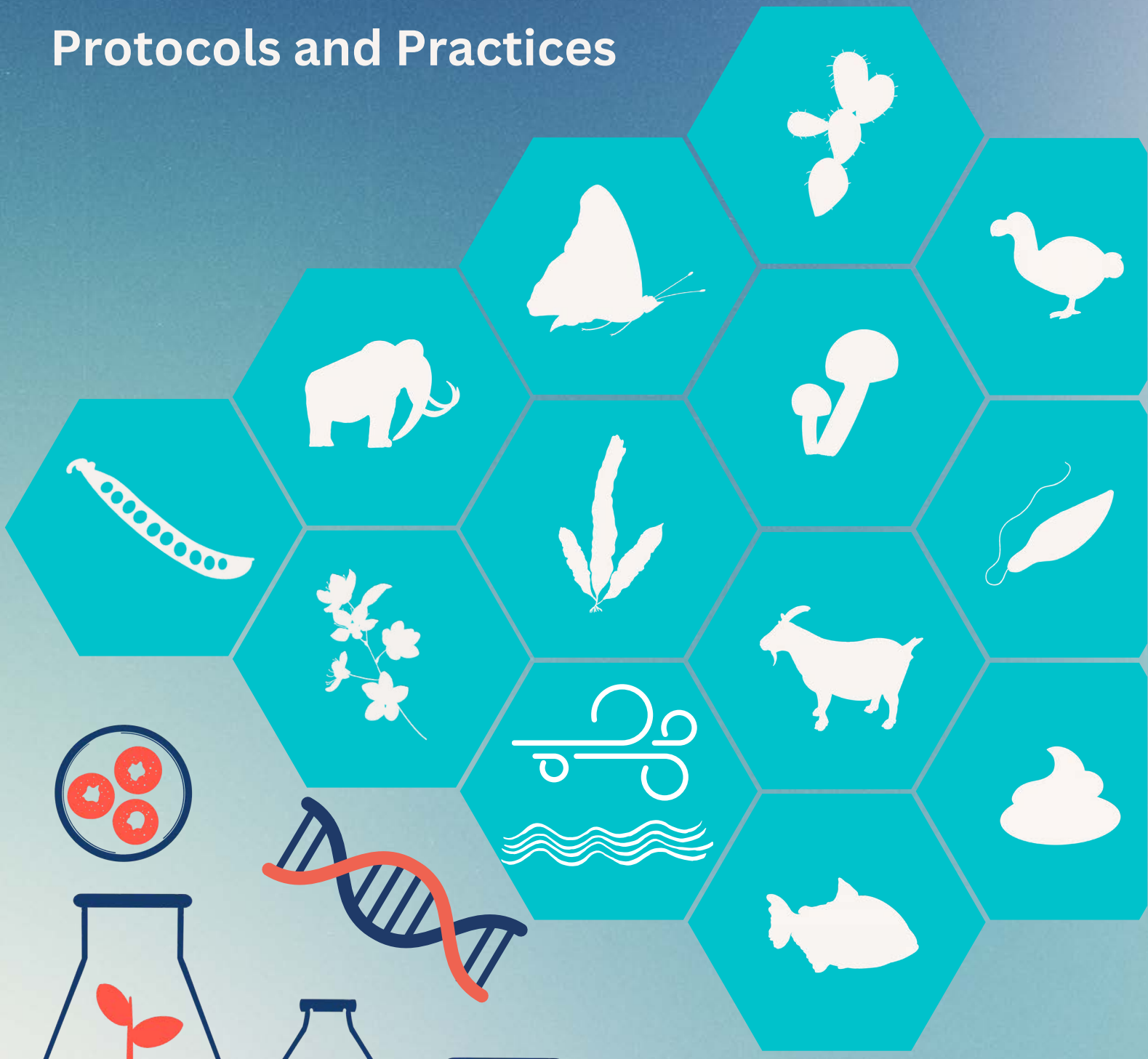
Biodiversity Biobanking

A Handbook on Protocols and Practices



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Biodiversity Biobanking – a Handbook on Protocols and Practices

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PENSOFT

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SYNTHESYS+
Synthesis of Systematic Resources
a DiSSCo project

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Preface

We are today confronted with an unprecedented, ever-increasing rate of global biodiversity decline at the ecosystem, the species, and the genetic level, with yet unforeseeable consequences for both our planet and humankind.

To mitigate the underlying anthropogenic processes, political action is overdue, informed by science. At the same time, the scientific community is called upon as a major player on another front: as a response to current and expected biodiversity loss and environmental degradation, we need to promptly and drastically ramp up efforts regarding *ex-situ* conservation and regarding the archival of molecular samples. Key infrastructures in this process are biobanks (see, e.g., Jarman et al. 2018; Ryder and Onuma 2018; Colella et al. 2020; Angeles and Catap 2022).

Biobanks are "future-making institutions" (Breithoff and Harrison 2020) and memory institutions at the same time, and they warrant the integrity, authenticity, availability, and (where necessary) confidentiality of molecular and/or viable samples and associated data for current and future research (Astrin et al. 2013).

Seed banks, DNA banks, culture collections, genebanks, genetic/biological resource centres, veterinary biobanks, parasite banks, germplasm banks, environmental specimen banks, etc.: biobanks (or biorepositories) come in a variety of forms, each of them playing an important role in the task to conserve and procure biodiversity or environmental samples. Together, their collections comprise (cold-)preserved samples from a multitude of environments and span the entire tree of life, in the form of whole organisms, or as fixed or viable subsamples.

To date, many biobanks still operate in a relatively isolated fashion. This does not necessarily imply that each biobank works for itself; extensive biobanking collaborations and networks exist (amongst others the World Federation of Culture Collections **WFCC**, or the Global Genome Biodiversity Network **GGBN**; or the overarching networks including human biobanking like the International Society for Biological and Environmental Repositories **ISBER**, the European, Middle Eastern, and African Society for Biopreservation and Biobanking **ESBB**, or the Asian Network of Research Resource Centers **ANRRC**). However, the biodiversity and environmental biobanking community is currently scattered into thematic 'biobanking tribes', with limited exchange amongst them. To some degree, this results from divergent near-term goals and from the particularities of the many different targeted organisms or sample types and their respective methodologies and metadata microcosms (Bach et al. 2012). Nonetheless, focusing on commonalities and further increasing the exchange and cooperation amongst biobanking tribes would be helpful in identifying research and taxonomic/geographic gaps, in harmonising data visualisation (e.g., Droege et al. 2014), in obtaining funding and optimising protocols and best practices.

The last aspect—development, optimisation and sharing of protocols and biobank practices—constitutes the focus of the present handbook. One important characteristic of biobanks is that they follow standardised workflows (Hewitt and Watson 2013).

We compiled extensive information on such workflows from throughout most of the biodiversity and environmental biobanking communities. Publications, grey literature, and Internet sources were reviewed, and proven experts consulted. By linking to protocols and practices from many different types of biobanks we hope to inspire interdisciplinary approaches and interconnect biobankers, and to serve as an aggregated resource for incipient and thematically expanding biobanks. Maybe the compilation of practices can also contribute to processes of method validation and standardisation.

This handbook is the first document to unify detailed information on such a wide range of biodiversity and environmental biobanking domains, targeting protists, fungi (here pragmatically divided into micro- and macrofungi, as procedures for the former are often close to protists, for the latter to

plants), lichens, plants, and animals. We mostly excluded bacteria, archaea, and viruses so as to reduce complexity, and because various comprehensive sources already exist for them (e.g., Lapage et al. 1970; Tedeschi and De Paoli 2011) We also deliberately excluded a single animal species: *Homo sapiens* (except for archaeological remains, etc.). Most practices relevant to human biobanking can be found in the ISBER **Best Practices: Recommendations for Repositories** (ISBER BP) and various ISO standards. The freely available ISBER BP are periodically updated (Campbell et al. 2018) and contain ample information and recommendations on biobank management, equipment, safety, quality control, and liquid nitrogen handling. They are edited with the ambition to serve as a resource to all types of biobanks and in fact, biodiversity biobanks often successfully orient themselves at the ISBER BP. However, they mostly lack specialised biodiversity and environmental information, e.g., regarding culture collections, seed banking, or environmental samples.

In this handbook, we aim to provide guidance and recommendations on field sampling, preservation, and storage of biomaterials along with management procedures. Chapters 4 to 7 focus on tissue and cell preservation and storage, whereas chapters 8 to 11 are concerned with DNA.

Whereas information on molecular methods that we collected may have a relatively short life cycle, we expect that particularly the information on culture methods (organismic and cellular) and on viable storage (of cells, propagules, and organisms) will remain up to date for a considerable time. This handbook should be used alongside species- or group-specific information to customise or improve protocols.

We would like to end on the note that biobanks are part of a much wider landscape of collections that together support life science research and possess great potential in helping to face the current biodiversity crisis. Biobanks are not isolated collections but exist in a nexus of other sample types and data—although the challenge remains to actually interconnect and semantically unlock them. Much like building bridges between the various biodiversity and environmental biobanking tribes, biobanks need to be aware of and interact with those collections that do not focus on molecular or viable samples.

Jonas J. Astrin & Carolina Corrales

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List of abbreviations

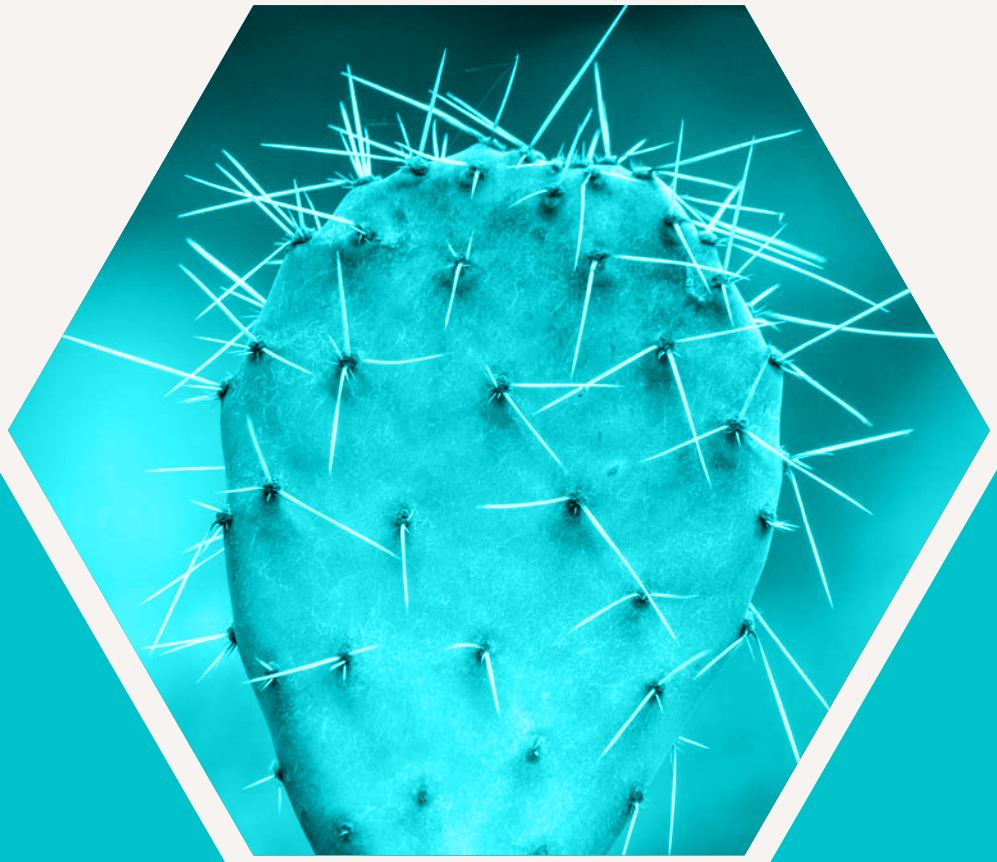
ABS	Acrylonitrile Butadiene Styrene
aDNA	Ancient DNA
AFLP	Amplified Fragment Length Polymorphism
APS	American Phytopathological Society
AQUAEXCEL	Aquaculture infrastructures for Excellence in European fish research project
ARS	Amphibian Ringer Solution
ART	Assisted Reproductive Techniques
ASSEMBLE	Association of European Marine Biological Laboratories Expanded project
BOLD	Barcode of Life Data System
CABI	Centre for Agriculture and Bioscience International, UK
CASA	Computer Assisted Sperm Analysis
CBOL	Consortium for the Barcode Of Life
CCAP	Culture Collection of Algae and Protozoa
CCDB	Canadian Centre for DNA Barcoding
CBD	Convention on Biological Diversity
CEN	European Committee for Standardisation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CGRFA	Commission on Genetic Resources for Food and Agriculture
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CMFDA	Chloromethylfluorescein Diacetate
COX	Mitochondrial Cytochrome c Oxidase gene
CPC	Center for *Plant Conservation*
CSH	Cold Spring Harbor
CTAB	cetyltrimethylammonium bromide
DAPI	4'6-diamidino-2-phenylindole
DBCA	Department of Biodiversity, Conservation and Attractions, Australia
ddPCR	Droplet digital PCR
DESS	DMSO/EDTA Salt-Saturated
DIN	DNA Integrity Number
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECACC	European Collection of Authenticated Cell Cultures
eDNA	Environmental DNA
EDTA	Ethylenediaminetetraacetic Acid
EMbaRC	European consortium of Microbial Resources Centres
EuReCa	European Regional Cattle breeds
DNA	Deoxyribonucleic Acid
ERFP	European Regional Focal Point for Animal Genetic Resources
ESCONET	European Native Seed Conservation Network
EUGENA	European Genebank Network for Animal Genetic Resources
FAANG	Functional Annotation of Animal Genomes project
FAO	Food and Agriculture Organization of the United Nations
FDA	Fluorescein Diacetate
FIMS	Field Information Management System

FTA	Flinders Technology Associates
Globaldiv	Global View of Livestock Biodiversity and Conservation Project
HMW	High-Molecular-Weight DNA
h	hour/s
HTS	High Throughput Sequencing
IDEM	Indiana Department of Environmental Management
IETS	International Embryo Technology Society
IMAGE	Innovative Management of Animal Genetic Resources Project
IPC	Internal Positive Controls
ISBER	International Society for Biological and Environmental Repositories
ISO	International Organization for Standardization
ITPGRFA	International Treaty on Plant Genetic Resources for Food and Agriculture
ITS	Internal Transcribed Spacer
IVC	*In Vitro* Collecting
IVF	*In Vitro* Fertilisation
LIMS	Laboratory Information Management System
LN2	Liquid Nitrogen
MHC	Major Histocompatibility Complex
min	minute/s
mtDNA	Mitochondrial DNA
MSBP	Millennium Seed Bank Partnership
NCBI	United States National Center for Biotechnology Information
NGS	Next Generation Sequencing
NP	Nagoya Protocol
nrLSU	Nuclear Large ribosomal Subunit
nrSSU	Nuclear Small ribosomal Subunit
NWFHS	National Wild Fish Health Survey
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate Buffered Saline
PCI	Phenol/Chloroform/Isoamyl alcohol
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PGC	Primordial Germ Cells
PI	Propidium Iodide
PTB	N-Phenacylthiazolium Bromide
PVP	Polyvinylpyrrolidone
PVS2	Plant Vitrification Solution 2
qPCR	Quantitative real-time Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic *DNA*
RFLP	Restriction Fragment Length Polymorphism
RH	Relative Humidity
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT-PCR	Real-time PCR Polymerase Chain Reaction
sedaDNA	Sedimentary ancient DNA
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
sp. / spp.	Species (singular/plural)

ssDNA	Single-stranded DNA library
STR	Single Tandem Repeat
TCEQ	Texas Commission on Environmental Quality
TTC or TZ	Triphenyl Tetrazolium Chloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
uHMW	Ultra High-molecular-weight DNA
UPA	Universal Plastid Amplicon
USDA	US Department of Agriculture
UUID	Unique Universal Identifier
WFCC	World Federation for Culture Collections
WGS	Whole Genome Sequencing

CHAPTER 1
Biobank Management

Carolina Corrales, Frederik Leliaert, and Jonas J. Astrin



Introduction

Biodiversity and environmental biobanks, which can stand by themselves or can be housed at e.g., natural history collections, botanical gardens, zoos/aquaria, or culture collections, are essential infrastructures not only to preserve and provide samples from different groups of organisms but also to sustain innovation, food security, natural resource management, biotechnology, and biological research (Hanner and Gregory 2007; Martins and Lima 2015; González et al. 2018; Bajerski et al. 2021). Defined criteria and goals when developing and establishing biobank workplans are important, along with the necessary financial means and human resources to carry out an efficient operation (Sackville Hamilton et al. 2002; Engels and Visser 2003). Harati et al. (2020) provide recommendations for starting a biobank, including equipment, standard operating procedures (SOPs), and funding.

Biobanks should establish in advance the purpose of their collections and organise them according to expected use. There are various collection categories, and biobanks may consist of one or a combination of them. A base or core collection is stored for the long-term for conservation purposes and is not used for distribution, whereas a working collection may be used for research, distribution, or breeding plans. An active collection, found mainly in seed and clonal plant biobanks, is basically a duplication of the core collection, and it is used for characterisation and multiplication (Painting et al. 1995; Dulloo et al. 2004).

This chapter briefly describes some key aspects in managing a biobank. Further detailed information regarding other topics not covered within this handbook (e.g., facility conditions, accessibility, shipping, etc.) are described in various best practice guidelines for repositories, such as the OECD (2007a), FAO (2012; 2014), ISBER (2018), the ISO 20387 (2018), and the WFCC (2010). These standards provide technical, legal, and ethical recommendations and general requirements for the establishment and maintenance of biobanks. Other standards, such as the ISO FDIS 21899 (2020) or the ISO 21709 (2020) focus on validation procedures in the context of biotechnology operations. Biobanks often follow any of the mentioned guidelines or create their own set of SOPs, as high-quality standards should be met to collect, process, store, use and distribute biological samples, without affecting sample quality, functionality, and integrity. An extensive overview of the requirements for biodiversity biobanks regarding governance, infrastructure, consumables, quality control, legal compliance and training can be found in Biodiversity Biobanks South Africa (2021).

Collection processing, inventory control, quality management, database maintenance and development, and sustainability preparation are typical responsibilities when managing a biobank (Vaught 2019). Hence, biobanks should allow for sufficient personnel to carry out these tasks. The curator and technical/professional staff for sample preservation and data curation should be appropriately trained and qualified (Shivas et al. 2005; FAO 2012). Ideally, training tools, such as online training material or capacity building workshops should be developed (e.g., sample collection and initial preservation, live tissue preservation, disease and zoonoses, preservation and storage methods, equipment use, quality control) (Fulton and Kresovich 2004; Bartels and Kotze 2006).

Development and update of the collection

Collections usually grow by directly collecting in the field, exchanging material with other collections or by receiving residual samples from research projects, animal healthcare or sanitary check-ups (Ortiz and Engels 2004; Leon-Quinto et al. 2009; Walter et al. 2020). Note, however, that DNA integrity and cell viability of these samples are affected by the initial preservation procedure prior to deposition in the collection, potentially reducing their utility in research (Zimkus and Ford 2014). Livestock biobanks usually strive to capture genetic changes over short

time spans by continuously sampling breeds (e.g., every 4–7 generations in cattle), as differences between the bank collection and the *in-situ* populations may arise (Blackburn 2018).

Extracting DNA from all samples should be a fundamental component in any collection, as this procedure can help not only to establish core collections but also to assess / characterise the genetic diversity of the collection (Ortiz and Engels 2004; Kang et al. 2006).

Note that adding more accessions to the collection and genetically characterising the collection are dependent on the biobank´s financial resources. The size of a collection may be limited by choosing to cease receiving new accessions, or discard existing accessions (Sackville Hamilton et al. 2002).

Genetic duplicates/clones or redundancies may generate overrepresentation in collections, especially those that store microorganisms and agricultural plants (e.g., *in vitro* or in field genebanks), leading to higher maintenance costs (Painting et al. 1995; Sackville Hamilton et al. 2002; Ortiz and Engels 2004; Smith et al. 2008; Uhlir 2011). These replicates may be stored for the short or medium term, or can become archival but, eventually, unwanted duplicates should be excluded from the collection (Keller et al. 2012; McCluskey 2011). Genetic characterisation can contribute to prompting this procedure, allowing the curator or manager to make well-informed decisions (Dulloo et al. 2004). Stevenson and Jong (1992) and the UKNCC (2001) suggest that only microbial strains that are identified and authenticated along with samples not yet represented in the collection should be kept to avoid unnecessary duplication. Further details regarding accession management of clonal propagules and landraces can be found in Sackville Hamilton et al. (2002). For the biobanking of wild animals/plants, a certain number of replicates is usually desired to assess genetic variability.

Collection backups

Traditionally, backups in biobanks are generated by taking subsamples/aliquots of a sample and storing them in separate freezers. However, there are some potential risks associated with mechanical freezer breakdowns (Hanner et al. 2005) and, especially, with energy blackouts (Muller et al. 2016). The safer option, and if resources allow, is safety duplication, which relies on the storage of a duplicated collection in a geographically separated location (e.g., mirror bank), to safeguard it as backup in case of physical disaster (Uhlir 2011; FAO 2012; CPC 2019; Priyanka et al. 2021). Associated sample data must also be deposited together with the collection duplications. The **CPC** (2019) provides recommendations for seed backups. Note that backup procedures may require legal agreements between the depositor and the mirror bank.

In addition, animal germplasm can be backed up by storing somatic cells (FAO 2012). In plants (e.g., clonal crops), complementary backups are maintained in the field, *in vitro*, or cryopreserved (Dulloo et al. 2004; FAO 2014; Acker et al. 2017; Panis et al. 2020). If field genebanks are used as backups, it is necessary to develop field maps, in case field labels are lost or destroyed (FAO 2014).

Risk management

Contingency plans and solutions for emergencies (e.g., natural disasters, pandemic situations, war, civil unrest) and breakdowns are needed to be developed to safeguard the collections. These plans usually include the establishment of a minimum number of staff, alternative sources to acquire supplies (e.g., LN2, laboratory consumables), logistics and communication (FAO 2012; Allocca et al. 2020a 2020b). Biobanks should follow specific guidelines, such as the ISBER (2018), ISO 31000 (2018), and the ISO IEC 31010 (2019). Akyüz et al. (2021) provide a catalogue of potential risks in human biobanking that equally apply to biodiversity biobanks. Regarding risks inherent to the biobanking process itself, Bajerski et al. (2021) described potential microbial cross contaminations of samples in LN2 storage tanks and developed a risk assessment spread-

sheet to help reduce this threat. Furthermore, Day et al. (2022) provided recommendations for emergencies involving cryostat failures and the potential implications of sample loss. Risk assessment is an active practice and hence, requires regular reviews and updates (FAO 2012; Akyüz et al. 2021).

Additionally, backups and integrity checks of data associated with the samples and with the biobank management are crucial in case of software failure or unexpected catastrophic events. A proper documentation system is a requirement in any biobank for an effective sample use (Ortiz and Engels 2004). Databases are essential components to biobanks not only because they include sample metadata but also because they allow the user to track changes (e.g., in nomenclature variables) and store any type of file, such as documents, digital images and videos (Shivas et al. 2005).

Biosafety and biosecurity

Human safety (i.e., avoiding biological, chemical, and physical hazards) is a crucial issue and many standards exist regarding the potential risks associated with biobanking activities (Atherton et al. 2017). Researchers should be especially aware of pathogens associated with animal samples both in the field and in the laboratory. In addition, microbial collections should provide proper infrastructure and high standards for material exchange/sharing (WHO 2021), along with prevention of exposure or accidental release of pathogens (Smith et al. 2017). Further regulations can be found in CABI (1995).

When dealing with livestock or animal products, biosecurity and zoonotic control measures should be taken. Some biosecurity guidelines have been developed to minimise the risk of zoonosis transmission during transportation of research animals (National Research Council 2006) or at various facilities (Australian Veterinary Association 2017; AAHA – American Animal Hospital Association 2018). The **OIE** (world organisation for animal health) also provides information on quarantine and surveillance of veterinary material. Refer to Wildlife Health Australia (2011, 2018) for guidelines on wildlife and zoo biosecurity. There are also risks and potential hazards associated with human remains, and hence, biosecurity measures should be considered. Antoine and Taylor (2014) and the Society for the Museum Archaeology (2020) provided possible risks and how to handle them.

Biobanks dealing with plant material should certify that plants and seeds are pest- and pathogen-free. The European and Mediterranean plant protection organisation (EPPO) developed several **standards on phytosanitary measures**. When dealing with plant pathogens, biobanks are required to maintain pathogens in biological safety cabinets, with restricted access to autoclaves, incubators, and freezers, and ideally, laboratories should not have windows to avoid potential pathogen releases. Further regulatory safety aspects for plant pathology can be found in Babcock et al. (2007).

Biobanks, especially those holding microbial collections, must be aware of any misuse or theft of the stored biological material (Smith et al. 2017). Risk assessments and accessibility always need to be considered. The OECD (2007b) developed standards specifically on biosecurity for biobanks, dealing with hazardous biological resources. Stalpers (2010) and Smith et al. (2017) provided guidance documents for safe practices, a summary of important legislations, and a list of reliable online resources describing biosecurity issues.

Quality management for repositories

Biobanks can implement a quality management system (QMS) to obtain an accreditation that ensures that the biobank workflow is conducted in accordance with standardised procedures and includes processes for improvement (Davis et al. 2012; Smith and Ryan 2012; Martins and Lima 2015; González et al. 2018). Among biodiversity biobanks, this is most frequently implemented in biobanks focusing on

microorganisms. The process of certification needs dedication of staff and resources, as well as the formulation of several documents, including SOPs, quality control manuals, management and planning programs, data/records management standards (González et al. 2018).

The most common certification is the ISO 9001 (2015), which provides a framework for any type of organisation to describe and record processes effectively (Martins and Lima 2015). It also defines general requirements for QMS by following the *plan - do - check - act* (PDCA) management approach (Perugini et al. 2018) that can help to preserve and control the operation´s proficiency, as well as to facilitate training of new staff (Martins and Nelson 2015). Further contributions to culture collections´ QMS that are not covered in the ISO 9001 can be found in Martins and Lima (2015) and Martin et al. (2015).

ISBER has developed a **self-assessment tool** for biobanks to help not only to identify how well best practices (e.g., ISBER and ISO 20387 standards) are followed but also to determine areas of improvement.

CHAPTER 2
Metadata and Data Management

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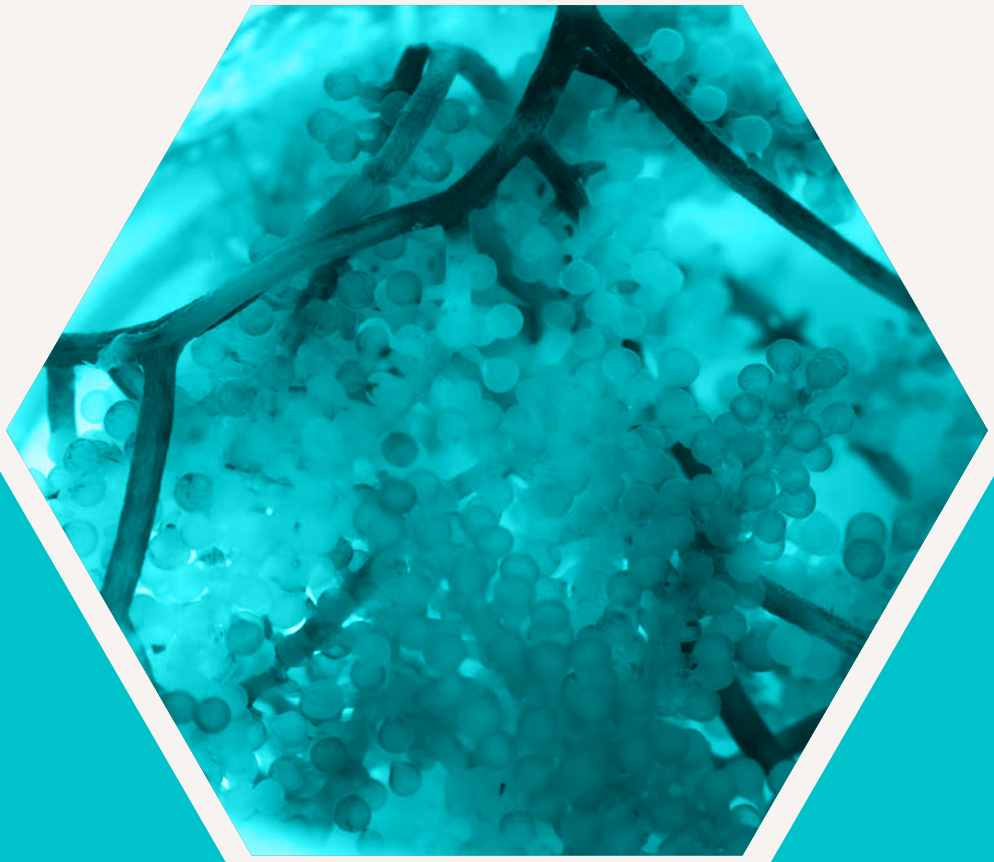

Introduction

Metadata or sample associated data are crucial and required for the processing and accessioning of specimens into collections (Martinez-Goss and Liao 2020). Collected biological material with incomplete or missing associated information lessens its scientific value, which may render it useless for research (Bennett 1999; Tomović et al. 2002; Martinez-Goss and Liao 2020; Damerow et al. 2021). Hence, collectors must have the skills and experience to record sufficient data when collecting samples for genomic analyses (Sackville Hamilton et al. 2002; ENSCONET 2009; FAO 2014).

RECOMMENDATION:
Databases contain sensitive sample and user data. Therefore, it is essential that databases are set up on computers connected to firewall-protected, internal servers. Computers and databases should be password-protected (Dolle et al. 2020), and all users should be registered. Consider accession rights and data protection issues (e.g., personal data, vulnerable wildlife populations).

Metadata comprise quantitative, qualitative, spatial, and imaging data that must be properly collected, analysed, and stored in a database for easy retrieval. This information will be useful for the design of field data forms and labels used in the field (Vanesh et al. 2010). Common collection database management software include **Specify**, **Arctos**, **Symbiota**, **GeOMe**, **PlutoF**, **BreedBase** and **Diversity Workbench**. Commercial management software include **Vernon Systems** and **Axiell**. Alternatively, a database can be started from scratch using open-source database management systems such as PostgreSQL, MySQL or MariaDB. It is crucial that the content of collection databases is regularly enhanced via **biocuration** (i.e., the integration of biological information into a database) to maintain data accuracy and reliability (Howe et al. 2008). The principles for curation of databases can be found in Odell et al. (2017).

RECOMMENDATION
The use of relational databases is preferable to using spreadsheets (e.g., Excel) when adopting a database system.

Ideally, metadata and their management should be determined and defined during the early stages of biobank or project planning to ensure that data collection procedures will be harmonised (Vanesh et al. 2010; FAO 2014; Lawniczak et al. 2022a). Data should follow the FAIR principles (Findability, Accessibility, Interoperability, and Reusability) as a first step towards standardisation for data exchange (Wilkinson et al. 2016). Some guidelines are already in place for microbiome data (Vangay Pajau et al. 2021), biodiversity genomics data (Lawniczak et al. 2022b), and environmental sample metadata (Berry et al. 2021; Damerow et al. 2021; Rimet et al. 2021). Furthermore, Hackett et al. (2019) provided a workflow of how to collect, share, use and validate biodiversity data from various sources.

Metadata should comply with the **Darwin Core format standard**, the **ABCD** (Access to Biological Collection Data 2005; Holetschek et al. 2012) standard, the Multi-crop passport descriptors (Alercia et al. 2015), or the **GGBN** data standard (Droege et al. 2016) to make data management consistent and compatible. (Rimet et al. 2021; Lawniczak et al. 2022b). The **Genomic Standards Consortium** also provides information regarding standards for genome and metagenome sequences. Moreover, it is highly recommended to include high-quality images of the specimens and make them publicly available (Lawniczak et al. 2022c).

So far, the reuse of data remains challenging not only because of limited application of data standards, but also because sample identifiers are often not consistent along the pipeline of sample processing and use (e.g., sharing of

samples among different laboratories or researchers, who rename or report different IDs in publications), thus impeding data tracking and scientific discovery (Chase et al. 2015; Damerow et al. 2021). Additionally, the use of a unique universal identifier (UUID) or globally unique identifier (GUID) is essential to enable identification of the original sample and its metadata (Triebel et al. 2018). Note that subsampled tissues, viable cells, and derivatives (e.g., DNA) should also have unique tissue identifiers, in addition to the source specimen ID, to define sample uses (Deck et al. 2012). Best practices for publication of biodiversity data management can help to integrate data between projects, allowing reproducibility (Costello and Wieczorek 2014).

RECOMMENDATION
Metadata must be linked both to voucher specimens/tissues/cells/extracts, and to genomic data via GUIDs/UUIDs. Note that GUIDs and UUIDs are synonyms and have the same meaning.

Sometimes, metadata associated with collecting events are initially recorded on Field Information Management System (FIMS) servers (Deck et al. 2012). FIMS or any other type of data collection (e.g., spreadsheets, notebooks, web forms) should include common, basic metadata, which are recommended for all collected samples, and which apply to all taxonomic groups:

- Unique collection number (e.g., UUID, collector´s number)
- Taxon name
- Collectors' name(s)
- Additional collectors
- Identifier's name (if different from collector)
- Identification date and identification´s method (e.g., barcoding, taxonomic keys)
- Date of collection [ISO standard 8601:2004(E)]
- Time of collection
- Geographic location (country, state, province, closest village, directions and distance to collection site, physical landmarks)
- GPS coordinates (in decimals) and datum (e.g., WGS84), including accuracy
- Elevation/depth
- Habitat
- Additional conditions (e.g., slope of ground, weather)
- Collecting method
- Life stage (if applicable)
- Organism part and amount of material
- Number of samples or duplicates
- Preservation and storage information
- Permit information
- Project data
- Image/sketch

RECOMMENDATION
Collecting numbers or UUID should not include dates, identifiers (collecting or field numbers, UUIDs/GUIDs) or any semantic at all (e.g., taxon names, collector names, storage information), as these data are subject to change and it may cause problems in databases, transcription errors and misleading results.

Other metadata regarding protist and fungal culturing (e.g., growth temperature, maintenance methods, phenotypic characteristics, dates the substratum was plated out and species presence), as well as publications and Nagoya Protocol compliance are usually updated at a later stage.

RECOMMENDATION
Ideally, database records should be linked with external taxonomic databases to track and automatically update changes in nomenclature (Robert et al. 2011).

Laboratory Information Management Systems (LIMS) are databases designed to maintain and to track, in a centralised manner, the different workflow stages concerning sample processing, experimental procedures (e.g., physiological tests, DNA analyses), and handling (Vu et al. 2012; Dolle et al. 2020). This information

Table 1. Secondary metadata for each group of organisms should also be recorded to describe the organism´s morphology and collecting conditions.

Organism group	Metadata
Microorganisms	Substrate
	Strain status type (e.g., neotype, epitype)
	Strain designation
	Transfer numbers
	Isolator´s name
	Date of isolation
	Pathogenicity
Fungi	Description of fungus (e.g., smell, colour, shape, size, diameter, indumentum)
	Substrate
	Microhabitat
	Associated vegetation
Algae	Littoral zone
	Type of algae (e.g., epilithic, epidendric)
	Tide
	Growth habit
	Relative abundance
	Substrate
	Water temperature
	Topography
	Water clarity
	Associated fauna
Plants	Vernacular name (local name in original language)
	Description of plant (e.g., smell, colour, growth form, fertility)
	Phenology
	Habit
	Abundance
	Associated species (e.g., pollinators)
	Substrate
	Exposure
	Soil type
	Water depth (for aquatic plants)
	Soil texture
	Land use
Animals	Sex
	Body measurements
	Ectoparasites
	Condition (e.g., dead, alive, decomposed)
Livestock*	Studbook number (for captive/bred species)
	Zoo/aquarium name

Organism group	Metadata
Livestock*	Donors Breed Breeder number Registration number and ID number Property rights Phenotype Management (e.g., intensive, mixed farming, extensive)
Parasites	Host species Site of infection Count number Storage vial
Plant pathogens	Identification method Host species Host damage (e.g., symptoms) Prevalence Management history (for plantations)
eDNA Airborne Soil/sediment Water	 Indoor/outdoor Average temperature Relative humidity Wind speed Wind direction Ground features Horizon pH Moisture Light Filter size/material Secchi depth
Museum specimens	Name of institution Museum ID (catalogue number) Type status

* The EFABISnet project has developed a documentation system especially designed for livestock (Duchev et al. 2010). EMbaRC (2012) has developed guidelines specific for microbial material.

can be easily shared with third parties or collaborators (Dolle et al. 2020). Several LIMS have been developed for specific uses. For instance, CASCADE is a management system created for ancient DNA experimental workflows (Dolle et al. 2020), HADB for Hawaiian algal diversity (Wang et al. 2009), CWRIS for crop wild relative information (Kell et al. 2007), and A-GRIN for animal germplasm resources (Blackburn et al. 2019). Among the more generic (open source or commercial) LIMS (Parker et al. 2012) are:

- **Species360**
- **Geneious/Biocode**
- **eLabFTW**
- SeqDB (Bilkhu et al. 2017)

- **BioloMICS**
- GOTIT (Malard et al. 2020)

LIMS developed for sequence data, especially for DNA barcoding, should include information about DNA extraction, PCR (e.g., primers, cycler programmes), and sequencing methods (e.g., sequencing platform, sample plate position, sequence data, coverage) and linked to the samples (Vu et al. 2012) to enable the examination of successful protocols and troubleshooting.

Moreover, LIMS include functions for tracking the physical storage of not only the voucher specimens but also of molecular vouchers, i.e., the corresponding tissue subsamples and by-products (e.g., DNA, RNA, HTS libraries) (Bilkhu et al. 2017). Note that it is also essential to record on LIMS how the samples are stored and the quantity of available material. This information will be important for loan procedures.

If FIMS and LIMS databases are used for sample traceability, they should be linked to each other or to a main database. However, it can be challenging to integrate all databases because data often need to be manually exported and reimported into each system. The BioCASe (**Biological Collection Access Service**) protocol was developed to aid discovery and retrieval of data between distributed databases (Holetschek et al. 2012). However, far more important than these technical solutions, is to store samples and their derivatives using GUIDs/UUIDs**.**

Field labelling

Field labels should include an identifier that links the material to the collected data. This identifier is often the collector's name or initials together with the collecting number, although the use of a barcode is becoming more common. Field labels should contain the collection number, the collector's name, date, location, project's name and species, if identified, as a minimum. Labels with fixed QR codes or barcodes can be produced and printed ahead using online resources (e.g., https://www.uuidgenerator.net/version4, https://qrcode.tec-it.com/de). The **Diversity Workbench platform** provides information about labelling preparation.

The use of thermal-printed labels, self-laminating labels featuring a barcode (linear and/or 2D) (e.g., Laser Cryo-Babies, Cryo-Tags, Scienova, PikaTAG), or vials with laser-etched barcodes (e.g., Nunc Bank-It, FluidX, NEST) should be preferred over manual vial labelling to avoid issues such as misspellings, illegible handwriting, or smearing, which can lead to sample mix-up or loss of data (Prendini et al. 2002; Dhargalkar et al. 2004; Altermatt et al. 2015; Lawniczak et al. 2022c). The FAO (2016) suggests that both vial and cap should be labelled, when doing so by hand (FAO 2016).

Labels are often placed inside the vials (Krogmann and Holstein 2010), but this practice can compromise the sample's molecular structure either directly or by affecting the preservation solution (Neumann 2010; Mengual et al. 2019). Laser-printed labels will not last in ethanol or generally over time, whereas thermal-printed polyester labels can damage the specimen; hence, they should not be used for this purpose (Krogmann and Holstein 2010; Mengual et al. 2019).

Triebel et al. (2018) suggested a workflow that facilitates data collection in the field including:

- The use of UUIDs either as a 2D code (e.g., QR), linear barcode or in human-readable format, both in notebooks and on labels.
- Preparation of labels and labelled containers before going to the field.
- Addition of descriptive information on labels, and photographs taken with a positioning system-enabled camera of the collection event per sample. Ideally, the specimen should be photographed together with the label to avoid potential mistakes that might arise downstream.
- Image processing after field collection is over.
- Curation and storage of data.

CHAPTER 3
Field Collection

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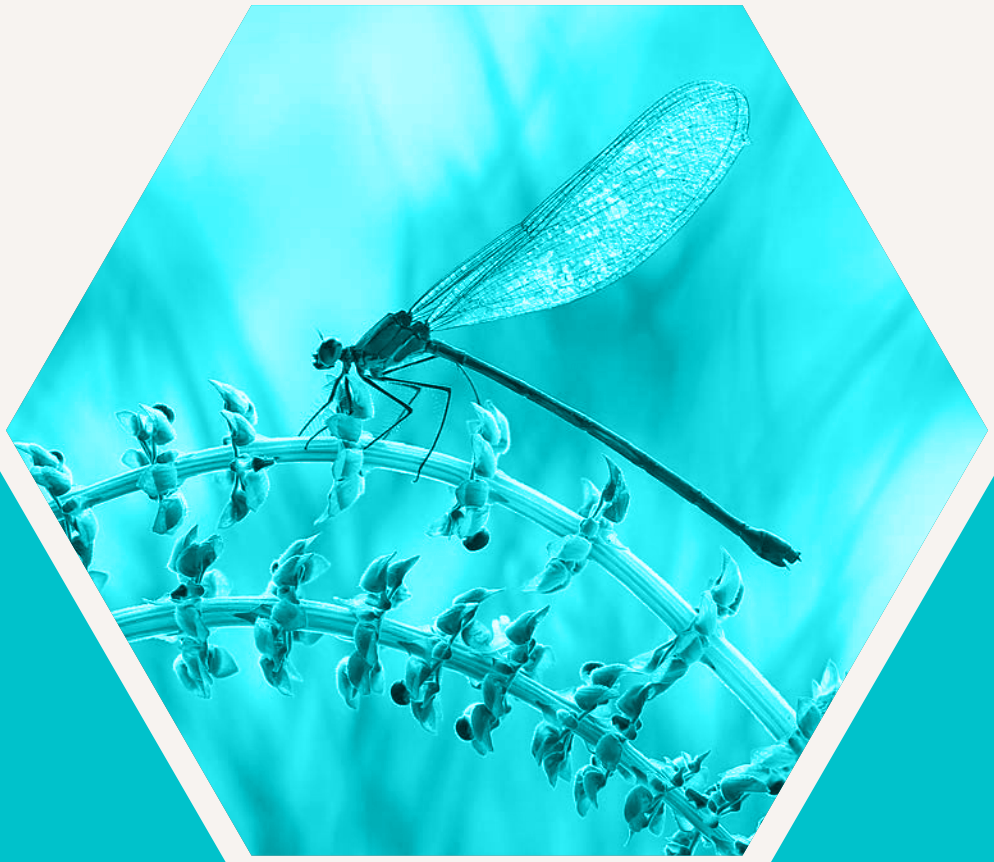

Before going to the field

For successful collecting, the methodology to be used, the timing and geographical location need to be carefully planned. Consider transportation, specialist equipment, and safety of all people involved. Furthermore, collectors should have the training, knowledge, and skills to carry out the field collection.

Permits and other documentation are generally required depending on the country and its legislation/policies prior to sampling. All collecting permits, including authorisation for collecting on private land, and genetic resource permits should be carried throughout the sampling period. If specimens are to be imported/exported, all national and international permits (e.g., CITES permit) stating the purpose of transaction should be obtained. Phytosanitary certificates are required to indicate that plant material is pest-free before it can be exported. Animal samples may be subject to veterinary inspection when entering the country of destination to minimise the risk of zoonoses (Ryder and Onuma 2018). Hence, attaching relevant notes may be helpful also for animal samples.

Data collection and photographs

Care should be taken to capture comprehensive data at the time of collection, including habitat, habit, abundance, accurate locality (GPS with uncertainty and datum) and description (see metadata chapter). Photographs of the sample should include a scale bar, colour chart and diagnostic details of structures that might deteriorate after collection, e.g., colour, shape, size. A standardised approach to taking photographs in the field will reduce the likelihood of missing an important characteristic.

RECOMMENDATION

Photography and documentation procedures should be implemented on all stages of sample collection and processing. Photos (including microphotography) will assist in identifying species and habitat conditions. An image archive will be easy to retrieve, review and compare with new specimens, when needed.

For macrofungi, macroalgae and lichens, photographs of the specimens should be taken in situ from various angles to capture all morphological aspects (e.g., structure of reproductive organs, whole thallus). Ideally, the entire collection process should also be documented. A detailed description should also be made, including aspects that may change after collecting or drying (e.g., colour, shape, odour, fungal veil presence), along with morphometric measurements (e.g., size, stipe length and width, cap diameter for macrofungi, meristem and blade thickness, blade area and sori area for seaweed). See Lodge et al. (2004) or Prance and Fechner (2017) for morphological descriptions of macrofungi. Chemical reactions in fresh should also be done in fresh specimens (e.g., colour changes in 5% KOH, FeSO4). Close-up photographs of plant and animal specimens should be taken, especially from those species that cannot be collected as vouchers, e.g., due to their endangered or rare status.

Data should be collected in field notebooks, data sheets (ideally with descriptions to tick), or in digital formats. The latter includes the use of free mobile phone apps such as EpiCollect – a data gathering platform synced with Google Cloud – (Glime 2017), or the **KoBoToolbox**, an open-source platform that curates information

and backs it up in real time (Meyer et al. 2021). Alternatively, Excel spreadsheets templates can also be used as FIMS to record data (refer to Deck et al. 2012, for detailed information). Digital data should be backed up regularly. If back-ups are not possible and there is a risk of the electronic data being lost or damaged, then paper copies of the raw data may be created. Note that spare batteries should also be taken to the field (ENSCONET 2009; Gemeinholzer et al. 2010; Vanesh et al. 2010) and that notebooks and data sheets should be stored in plastic bags to keep them dry (Glime 2017) and photographed for immediate digital backups. Some institutions will ask that paper notes are deposited in the repository together with the sample (Funk et al. 2017).

RECOMMENDATION
Ideally, a multi-disciplinary team with expertise in different areas should be available to collect reliable data during fieldwork. Confirm that all team members are using identical protocols.

Filed labels should be written as samples are collected, ideally using a pencil, waxed pencil, or permanent archival black ink (e.g., India ink) on waterproof paper (e.g., Rite-in-the-Rain paper) or synthetic, polypropylene paper (e.g., Kimdura or Tyvek) to avoid tearing (Prendini et al. 2002; Lodge et al. 2004; Neumann 2010; Gardner et al. 2012; Prospero et al. 2017). See further information regarding labelling in the metadata chapter.

Disposable gloves and tools should be changed after collection of each sample if there is a risk of contamination. Reusable equipment and tools can be sterilised using a series of ethanol-water baths followed by flame, or enzymes (DNases, RNases) (Gostel et al. 2016; Tim Fulcher, pers comm.), or can be soaked in 10% bleach and rinsed in DNase-free sterile water between sites. Furthermore, all interiors of bags used for storage must be clean.

Microorganisms

Microbiological repositories hold living collections that can be preserved and reproduced perpetually (McCluskey 2017). Most of these have been created to secure strains important for human health and industry (González and Jiménez 2013). Note that microbiological repositories and culture collections have to comply with many legislative regulations (e.g., biosafety, quarantine), affecting the distribution and use of these resources (Smith and Ryan 2019). Regulations and guidance are listed in Smith et al. (2001), González and Jiménez, (2013), and Smith and Ryan (2019). If patents are involved, the Budapest treaty must be followed (McCluskey and Wiest 2011; Saxon et al. 2020).

Specimens (e.g., living cultures and herbarium material) have to be deposited in a public repository to keep a representative available for validation, future studies, and subsequent reidentifications (Agerer et al. 2000; Shivas et al. 2005; Agarwal and Sharma 2006; Gachon et al. 2007; Abd-Elsalam et al. 2010; Stackebrandt et al. 2014; Warren et al. 2017). Unfortunately, publishing a record of the studied material or mentioning the institution where the material is stored is uncommon, impeding access and reproducibility (Agerer et al. 2000). Stackebrandt et al. (2014) suggest a set of criteria to facilitate deposition and release of microbial material into public collections and making this information public.

This section does not cover bacteria or archaea, as they are either well considered elsewhere (Lapage et al. 1970; Starr et al. 1981; Spring 2006; Tindall 2007; Tedeschi and De Paoli 2011; Gray et al. 2013; Peiren et al. 2015; Rose et al. 2015) or have overlapping with eDNA procedures described below. Sampling

methods of extremophilic microorganisms have been provided by Rainey and Oren (2006), and methods for atypical environments by Gurtler and Trevors (2018).

Protists

Protista is an informal, polyphyletic designation for eukaryotic organisms not belonging to multicellular plants, fungi, or animals, and thus cover free-living, predominantly unicellular eukaryotes that can be heterotrophic or autotrophic (Altermatt et al. 2015, for a more detailed definition see Burki et al. 2021). Algae are also considered within Protista (Gachon et al. 2013; Saxon et al. 2020). For convenience, only microalgae (e.g., euglenoids, dinoflagellates, diatoms) will be included in this section. The diversity and abundance within Protista have been largely underestimated by traditional approaches such as culturing and microscopy, due to the challenges to collect and identify species (Caron and Schnetzer 2007; Medinger et al. 2010; Foissner 2014). Hence, culture techniques should be improved to identify and characterise morphologically, genetically, and functionally the collected specimens (Geisen et al. 2018; Gooday et al. 2020). Environmental samples and molecular approaches, however, have been currently used, and it is now a standard, at least, for soil microbial diversity assessment (Geisen et al. 2018).

Altermatt et al. (2015) have provided the first steps to the workflow standardisation in protists, leading to the development of a **protocol repository**.

Collecting methods

Protists can be collected and isolated from water (Balzano et al. 2012; Gooday et al. 2020), sediment (Schoenle et al. 2021), and soil (Fiore-Donno et al. 2016; Ceja-Navarro et al. 2021). Some groups such as flagellates, ciliates, and naked amoebae, can be found in all substrata (Gooday et al. 2020). As soon as samples are collected, they should remain cool and dark and should be transported as soon as possible to the laboratory for further processing (Altermatt et al. 2015). Note that not only the environment, but also the type of protists—and their mobility mode—will define the sampling and isolation method to be used (Caron and Schnetzer 2007).

Water collection. Marine plankton can be collected using either plankton nets or water samplers (e.g., Niskin bottles, Ruttner, Schindler-Patalas samplers) or a combination of both techniques (Gifford and Caron 2000; Templado et al. 2010; Valdecasas et al. 2010; IDEM 2016; Flores et al. 2019; The Nansen Legacy 2021). Net mesh size will depend on the organisms to be sampled. A mesh size between 5 µm and 80 µm is used for nanoplankton (e.g., heterotrophic flagellates) and microplankton (e.g., ciliates and heterotrophic dinoflagellates), whereas a mesh size of 125–150 µm is needed for mesoplankton and 500 µm for larger plankton (Kraberg et al. 2017). Nets may destroy some fragile organisms, so they are usually preferred for collecting encased protists such as sarcodines and tintinnid ciliates (Gifford and Caron 2000). Note that nets may also become clogged (Kraberg et al. 2017).

Alternatively, phytoplankton and algal-bloom samples (e.g., red tide, golden algae, cyanobacteria) can be collected from surface water (0.3 m depth) using clean jars (200 ml – 1 l) (Gifford and Caron 2000; Franks and Keafer 2004; Caron and Schnetzer 2007; TCEQ 2014). The required sampled volume from freshwater bodies will depend on the water colour/transparency. A sample volume of 1 l should be sufficient for nutrient-rich waterbodies, whereas larger volumes are necessary for low-nutrient, unproductive waterbodies (Porter et al. 1993). The use of a Kemmerer or Van Dorn samplers should be considered when plankton is too abundant, or waterbodies are eutrophic (Tceq 2014). Ideally sample containers should be wrapped in aluminium foil to avoid light as much as possible. To avoid shearing and damage to the algae, samples should not be strongly shaken (Pintor and Vital 2020).

Refer to Leya (2020) and Johnstone et al. (2002) for detailed sampling, isolation, and

maintenance protocols for cryophilic algae. A protocol, including a video explanation, for conjugating algae can also be found in Tsuchikane et al. (2018). Water sampling methods to detect parasitic forms can be found in Hill (2015). Guidelines for sampling diatoms are provided in CEN (2014). Further protocols for plankton and benthic freshwater and marine samples are found in Lee and Soldo (1992).

For DNA analyses, samples should be filtered (see methods in eDNA section), filters carefully folded into quarters using forceps, with filtered organisms inside, wrapped in aluminium foil, and stored in a plastic bag at cool temperature (at least 4 °C) and examined within 24 h of collection (Šlapeta et al. 2005; Carim et al. 2016). For RNA analyses, flash freezing in LN2 is advisable. Otherwise, samples can be air-dried and stored at -80 °C until further processing (Flores et al. 2019; Medinger et al. 2010). Water samples can also be preserved in 97% alcohol, DESS, in RNAlater or similar solutions, or FTA cards (Warren et al. 2017; Bailet et al. 2019).

Sediment and soil collection. Several soil subsamples are usually collected at each sampling plot from the top layer (0–10 cm depth) with a shovel or a corer. Samples should also include litter and humus to maintain protist growth. Note that dormant stages of some microalgae species can also be recovered from soil (e.g., *Haematococcus pluvialis* or the cyanobacterium *Nostoc commune*) (Day 2007). Pebbles and roots should be removed (Fiore-Donno et al. 2016), and if necessary, soil can be manually crushed (Foissner et al. 2005). Samples should be air-dried for at least one month and sealed in plastic bags until further processing (Foissner et al. 2002). Otherwise, subsamples can be immediately sieved, mixed, homogenised, and frozen, if they are required for DNA analyses and not for culturing (Fiore-Donno et al. 2016).

A multicorer is commonly used to obtain sediment samples from the sea/ocean. Cores should be kept dark and cool until reaching the laboratory (Lee and Soldo 1992). Subsamples should be taken from the upper 2 mm of the core with a sterile syringe (Schoenle et al. 2021) or cores should be cut into different layers for subsampling (Hohlfeld 2021). Sediments from shallow ponds or other freshwater bodies can be collected using sterile Falcon tubes (Šlapeta et al. 2005). Protists can also be sampled and isolated from permafrost sediment cores following Malavin et al. (2020). Sediment samples can be fixed with 70% ethanol and stored at -80 °C or directly deep frozen at -80 °C (Schoenle et al. 2021).

Detailed protocols for sediment and soil samples, along with sampling collection on artificial substrata can be found in Lee and Soldo (1992). Further protocols for soil microbial sampling methods and for drilling, coring, and sampling subsurface environments can be found in Knaebel (2007) and Kieft et al. (2007), respectively.

Dormant stages of certain algae can be stored dried, either at cool or ambient temperatures. However, the viability of dried material will decrease with time. Aquatic species do not exhibit dormant stages; hence they are not suitable for drying (Kapoore et al. 2019).

Species identification and isolation

Live counting is crucial for identification since most protist species are determined by their movements. This step needs to be performed directly after collection because not only can species composition change with time (Arndt et al. 2000; Hohlfeld 2021) but ciliates may also encyst quickly (Lee and Soldo 1992). Previously, only fixation and staining were used, but these methods caused shape alterations, shrinkage, and loss of flagella, making identification rather difficult (Poynton 1994; Hohlfeld 2021). Ideally, both live and fixed counting should be performed for comparison. Many solutions can be used for fixation (Poynton 1994; Gifford and Caron 2000), but acid Lugol's iodine solution (2–10%) is the preferred method, as it is suitable for molecular analyses (Sano et al. 2020). Refer to Auinger et al. (2008) for identification of protists preserved in Lugol's.

Soil, sediment, and benthic water samples should be diluted (e.g., 1/5) with filtered or sterile water (freshwater or seawater, depending on

where the sample was taken). Pelagic water samples do not need filtering, but they need to be carefully agitated to minimise settlement (Gifford and Caron 2000; Weitere and Arndt 2003). For examination under a phase-contrast microscope, between 1–5 µl of the mix should be taken. A Sedgewick- Rafter chamber can be used for better counting (Arndt et al. 2000; Hohlfeld 2021).

Automated equipment such as the FlowCam or the FlowCytobot can be used for identifying, counting, and imaging marine protists. Note that such methods cannot replace manual counting (Kraberg et al. 2017).

Sometimes identification and isolation occur after culturing, especially for soil/sediment suspension samples (Foissner et al. 2002; Schoenle et al. 2021). Typically, 2–5 ml of percolated water from a tilted culture can be collected using a Pasteur pipette. Water should be sampled from various parts of the Petri dish. Around 100 µl are placed on a slide to be identified under a microscope (Foissner et al. 2005). Note that certain species may disappear due to competition or to artificial culture conditions (Hohlfeld 2021).

Isolation can be carried out by enrichment, dilution, and physical methods under a microscope (Acreman 1994; Caron and Schnetzer 2007; Day 2007) and ideally by using multi-dishes (e.g., microtiter plates) rather than microscopic slides (Hansen et al. 2020; Pröschold et al. 2021). Enrichment involves the inoculation and incubation of a sample either onto an agar plate (e.g., the streak plate method) or into a two-fold medium volume together with boiled grains that help to promote bacterial growth. Dilution refers to serial transfers of the sample into a medium and it is ideal for single protist cells. Physical methods comprise the use of micropipettes or Pasteur pipettes for selection of individual cells—usually large or slow—and their subsequent transfer to a medium (Esteban et al. 2015; **Department of Biological Sciences**, George Washington University). Step-by-step protocols are provided in Pintor and Vital (2020).

Alternatively, organisms that contain pigments giving off a fluorescent signal (e.g., phytoplankton) can be isolated directly from water samples using flow cytometry sorting (Surek and Melkonian 2004; Andersen 2005; Balzano et al. 2012; Esteban et al. 2015). Ciliates and flagellates, especially from sediment samples, can be isolated using electromigration, whereas amoebae can be isolated using agar plating methods (Esteban et al. 2015). Gentle sonication can be used to remove bacteria without damaging the algae (Surek and Melkonian 2004). Concentration of protists is usually done by centrifugation (Starink et al. 1994), sedimentation using Utermöhl chambers (Valdecasas et al. 2010; The Nansen Legacy 2021), or tangential flow filtration (Marie et al. 2010 or **protocols.io repository**; Balzano et al. 2012; The Nansen Legacy 2021).

RECOMMENDATION

Dilution is the most commonly use method to isolate and wash protists. Between five and ten individuals should be isolated to safeguard the species/morphotype growth. Single cells (a monoclonal population) can be maintained in an axenic (contamination-free) culture by adding antibiotics, unless the species is predatory, i.e., requiring other living forms to survive.

For further identification, enumeration, and isolation protocols, refer to Kemp et al. (1993). Detailed enrichment protocols and dilution protocols can be found in Lee and Soldo (1992) and Altermatt et al. (2015) respectively. A comprehensive and detailed manual has been developed by Hansen et al. (2020) for the isolation and cultivation of mixoplankton. Metting (1994), Andersen (2005), and the Nansen Legacy (2021) also provide protocols for the identification, isolation, and purification of microalgal cultures.

Morphological characterisation of isolates can be carried out using high-resolution video microscopy and electron microscopy (Schoenle et al. 2021). After sorting, samples should be immediately transferred to 4 °C (Balzano et al. 2012) or stored at -20 °C with or without absolute ethanol for molecular studies (Valdecasas et al. 2010).

RECOMMENDATION
An important practice is to avoid the spread/escape/disposal of protists from laboratory cultures into natural ecosystems by releasing them down the drain. All material used in the laboratory should be disposed of or sterilised, and organisms should be killed by heat or addition of chemicals (e.g., bleach).

Microfungi and fungus-like forms

This group of organisms is relatively understudied, and interest has mainly focused on its commercial and industrial applications (Buyck et al. 2010). Microfungi and fungus-like forms are not easy to collect, as they are either very tiny or live hidden in a substrate or within hosts. In most cases substrata are collected rather than the fungus itself. Special laboratory conditions for culturing are hence required for the isolation and identification of species (Buyck et al. 2010). Specimens have to be sampled together with the substrate and may not be visible, and they should be slowly air-dried (Wu Q et al. 2004; Agarwal and Sharma 2006). If substrata are not to be dried, they should be incubated and cultured within a day after collection (Spiegel et al. 2007). After drying, samples should be stored at temperatures as close to field conditions as possible (Wrigley de Basanta and Estrada-Torres 2017). In addition, many fungus-like organisms are obligate biotrophs and thus cannot be cultured separately from their hosts (Buaya and Thines 2020).

Collecting methods

Collection of mycetozoans, also called terrestrial amoeboid protists, include myxomycetes, dictyostelids, protostelids and acrasids. They are found on dead aerial parts of plants, rotting wood, bark of living trees, litter, and dung (Spiegel et al. 2004). Most fruiting bodies (i.e., the sporulating form) have to be detected by examining substrata with a microscope, whereas some fruiting bodies from myxomycetes can be seen with the naked eye or by using a hand lens (10×–20× magnification) (Buyck et al. 2010). Fruiting body specimens should be separated and cleaned with fine forceps, scissors or pruning clippers before placing them in cardboard boxes. Myxomycetes can also be found in the sclerotia form, which can be transferred to agar cultures or moist chambers for developing and subsequent species identification (Wrigley de Basanta and Estrada-Torres 2017).

Certain genera of myxomycetes can only be collected in the field (e.g., *Fuligo*, *Lycogala*, *Cribraria*) and, so far, just a few species can be kept in agar culture (Spiegel et al. 2004; Wrigley de Basanta and Estrada-Torres 2017). Incubation and culture techniques for mycetozoans are found in Spiegel et al. (2004). Mycetozoans can be isolated using the spore-touch technique, where spores are recovered with a sterile needle, and transferred to a Petri dish containing water agar and *E. coli* as a food source. Single-cell isolation should be achieved after the mycetozoan is in culture (Spiegel et al. 2004). Further recommendations are given in the **Beginner´s guide to isolating and culturing eumycetozoans**.

Note: Microfungi (i.e., small ascomycetes) and mycetozoans, associated with microhabitats, can be grown and isolated using the moist chamber technique. The substrate should preferably be cultured right after collection, but it can also be stored for up to a year after which fungi can still be obtained (Wrigley de Basanta and Estrada-Torres 2017). It is important to have the right temperature, humidity, and light conditions for a successful culture (Beales 2012).
In general, moist chambers are prepared using damp paper towels, Whatman number one filter paper, or water agar placed in a Petri dish (Krug 2004). Clear, tightly sealed plastic boxes with dry paper should be used when fruits or tubers are the substrata (Shivas et al. 2005; Beales 2012). An inflated plastic bag may also be used as an alternative (Beales 2012). The substrate is placed on top of this layer and water may

added for moisture if filter paper is used. The moist chamber will dry very slowly, due to the low air circulation given by the Petri dish lid. Ideally, dishes are examined under a microscope or stereoscope every day during the first week. Cultures can be maintained up to three months, during which rehydrating them using an atomizer is necessary, and direct sunlight should be avoided. Moist chambers can be kept at room temperature (20 °C), unless the samples come from a cold environment. Contamination by hyphomycetes and zygomycetes can be decreased by incubating moist chambers at ≤18 °C (Krug 2004). Once visible myxomycetes fruiting bodies appear, they should be removed with a microscalpel or fine forceps, and transferred to a dried, clean Petri dish to allow slow drying for 24 h. Other type of mycetozoans should be further subculture for identification (Wrigley de Basanta and Estrada-Torres 2017, **Eumycetozoan project**). Refer to Krug (2004) for equipment and chambers to be used with special fungal groups or situations.

Collecting techniques are determined by substrate type (Wrigley de Basanta and Estrada-Torres 2017):

Litter and wood collection. Visible mature fruiting bodies collected from aerial and ground litter, rotten wood and fresh bark should be placed in cardboard boxes (e.g., matchboxes) with the substratum glued or attached with a cork and pins to the bottom to prevent damage (Spiegel et al. 2004; Buyck et al. 2010). Bark pieces from living trees (ca. 2–4 cm each) should be carefully removed with a chisel, avoiding damage to the tree´s inner tissue (Wrigley de Basanta and Estrada-Torres 2017). According to Callan and Carris (2004), twig microepiphytes can be collected by „turning the twig against two razor blades mounted 3.3 mm apart in a wooden block; then the bark surface should be moistened, and the microepiphytes scraped off using a flattened probe". Bark should be allowed to fully dry, otherwise non-target fungi will overgrow. Boxes should be stored in a cool and dry place. If no fruiting bodies are seen, substrata should be placed in paper bags and allowed to dry. In the laboratory, substrata should be incubated in a moist chamber. Alternatively, dried litter leaves can be pulverised and washed with distilled water to remove spores using the particle filtration method. Filters are then placed on Petri dishes with agar (Schmit and Lodge 2005). Further protocols and recommendations are given in Cannon and Sutton (2004).

Soil collection. As soil fungi aggregations tend to have uneven distributions, random samples of ca. 20–50 g each should be collected from around the roots of vascular plants by scraping the soil and placing them in independent sterile Ziploc bags (Spiegel et al. 2004, **Eumycetozoan project**). Root tissue (25–100 g) may also be included if the main goal is to search for pathogenic fungi. Samples can subsequently be bulked together in a strong plastic bag, which should be tightly secured at the top (Lane 2012). Keep samples cool at 4–8 °C until processing. (Shivas et al. 2005). Do not sample soaked soil and do not leave soil samples in the direct sunlight.

The Cavender method, or serial dilution with sterile water, is the most common protocol for isolating soil fungi (Spiegel et al. 2004). Soil should be ground, mixed thoroughly with 10 ml water, and 1 ml of the suspension should be transferred to a tube containing 9 ml of water (Shivas et al. 2005). Two dilutions are usually enough. A small amount of the final dilution should be spread evenly on a Petri dish with agar medium. Plates should be left open until excess of water has evaporated (Spiegel et al. 2004). As soon as fungi appear, they should be subcultured to avoid overgrowth (Shivas et al. 2005). Suspension methods promote fungi that sporulate profusely, whereas soil-plate methods are better for mycelium fungi (Beales 2012).

Another alternative is to sieve soilborne fungi that produce sclerotia or sporangia. Refer to Beales (2012) for protocol.

Air collection. Oomycetes and fungal pathogens, such as rusts or mildews, produce airborne spores (Beales 2012; Levetin 2015)

that can be sampled following the methods mentioned in the eDNA section.

Freshwater collection. Oomycetes are collected from freshwater samples or from sediments. Samples should be kept cool and cultured within a few hours of collection. Samples should be diluted with sterilised distilled water and added to a deep Petri dish. Snakeskin squares (1 cm^2), sesame seeds, or rice grain are used as baits (Buaya et al. 2019; Martín et al. 2020). *In situ* baiting can also take place by suspending mesh bags in the water or burying them in the sediment for 1–2 weeks (Shearer et al. 2004). Facultative pathogens such as *Phytophthora* or *Halophytophthora* species, may also be induced to sporulate by floating infected plant material in Petri´s mineral solution or sterile pond water (Beales 2012; Bennett and Thines 2019). Further protocols for the isolation and incubation of *Phytophthora* are described in Drenth and Sendall (2001) and Martin et al. (2012). A detailed protocol for the sampling, isolation, and preservation of Saprolegniales is provided by Sandoval-Sierra and Diénguez-Uribeondo (2015).

Fungi, such as hyphochytriomycetes and chytridiomycetes, can be collected by sampling water and a bit of organic debris (ca. 10 cm^3) from the study site and placing them in a deep Petri dish (Shearer et al. 2004). The baiting technique is used for isolation of these freshwater fungi (Agarwal and Sharma 2006). Baits represent organic debris, usually made of chitin, cellulose, and keratin, and pollen grains. A bait mix should be added to the Petri dish, avoiding an excess of it. Cultures should be incubated at a similar temperature found at the collection site. Fungi will begin growing after 1–2 days of incubation (Shearer et al. 2004). Collection can also take place *in situ*, using nylon net bags containing wrapped pollen or from algae leaves and waterlogged wood. Refer to Shearer et al. (2004) for detailed protocols. Isolation can take place as soon as mature sporangia are present (Shearer et al. 2004).

Aquatic hyphomycetes can be collected either from stream foam samples or submerged decaying vegetation (Shearer et al. 2004). If leaves are collected, they should be placed in polythene bags or tubes together with water from the site (Agarwal and Sharma 2006). Leaves should be washed with sterilised distilled water and 10–20 disks should be cut with a hole puncher. Ideally, the petiole should also be included. Disks should fall into a Petri dish and be left to incubate at the same temperature as at the collection site for 2–3 days. Foam samples should be collected using glass or plastic jars, avoiding water take-up, so that conidia are not diluted (Shearer et al. 2004).

Freshwater ascomycetes can be sampled by collecting dead macrophytes or woody debris on the edge of waterbodies. Substratum samples should be placed into plastic bags containing paper towels and stored in a cooler box until reaching the laboratory. Then samples should be rinsed, and any mud, sand, and algae should be removed with a spatula. Samples should then be placed on moistened filter paper to be used as incubator chambers at room temperature (Shearer et al. 2004).

For identification, isolation and culturing protocols of freshwater fungi refer to Shearer et al. (2004).

Seawater collection. Fungi and oomycetes can be found in diverse types of substrata such as sea-ice, sea-foam, submerged wood, mangroves, sediment, algae, and animals. Ideally, samples of various decay phases should be collected and placed in an incubation chamber (Bennett and Thines 2019; Overy et al. 2019). Sea-foam can be collected using sterile falcon tubes (Overy et al. 2014). If sea-foam is dried, it can be scratched and resuspended in sterile seawater. Sea-foam should be processed right after collection, otherwise yeast will overgrow other fungi and oomycetes in the sample. Note that sea-foam will not only contain marine species, but also terrestrial ones (Overy et al. 2014). For further collection details and sample culturing, refer to Overy et al. (2019) and references therein. Maintenance of marine fungi cultures can be found in Nakagiri (2012). Culture methods for deep-sea fungi can be found in Nagahama and Nagano (2011).

Stone collection. Collection of microfungi and microalgae from stone surfaces has traditionally been done by obtaining stone chips

or flakes with a chisel (Cutler et al. 2012). Alternatively, sterile adhesive tape strips (e.g., Fungi-tape) (Cutler et al. 2012; Ding et al. 2020) or moistened filter paper can be used as a non-invasive method (Valdecasas et al. 2010). Tape strips should gently cover the stone for around 5 s. The tape´s waxed backing should be put back in place and the sample should be stored in a sterile plastic bag at -20 °C (Cutler et al. 2012). Alternatively, the tape can be immediately placed on sterile glass microscope slides, which should be kept in a box until arrival to the laboratory. Strips should be cut into small pieces (5 × 5 mm) for straight inoculation into a medium for fungi or algae (Urzì & De Leo 2001) or cut in 2 × 2 mm pieces for DNA analyses (Cutler et al. 2012). Biofilm samples can also be taken by softly scratching the stone with a sterile scalpel and placing them into sterile vials, also inoculated on agar medium (Petraretti et al. 2021). After the incubation period, the strains should be enumerated and isolated (Petraretti et al. 2021).

Fungi-tape has also been used on bone surfaces (Pinzari et al. 2020), wooden organ pipes (Štafura et al. 2017), gelatine-silver photographs (Sclocchi et al. 2016), and parchment (Pinzari et al. 2012). Simple clear adhesive tape is also an option, and it has been successfully used on leaves and tomatoes to sample bacteria (Bisha and Brehm-Stecher 2009), and fungi found on smooth surfaces such as tiles or walls (Yang 2003).

Microfungi can also be sampled by swabbing with sterile cotton swabs that can be used for inoculation (Pinzari et al. 2012). Another approach is to use HEMA cryogels, which can equally retrieve microorganisms from stone surfaces (Silva et al. 2022).

Dung collection. Droppings of herbivorous animals should ideally be collected with a trowel and stored in a plastic bag. Various fungi will be obtained, depending on the stage of dung decomposition (Agarwal and Sharma 2006). Mycetozoans can be sampled on dung no older than one day (Spiegel et al. 2004), whereas other coprophilous fungi can be found on later decomposition stages (Agarwal and Sharma 2006). If the dung is dry, it can be moistened with water. At the laboratory, samples are placed in moist chambers, made of damp paper towels placed in finger bowls, bell jars, or Petri dishes (Buyck et al. 2010). After one or two days, fruiting bodies will appear, and a succession of species will be observed for several weeks (Krug 2004). Delicate and ephemeral mycetozoans should be immediately cultured for further characterisation using the spore-touch method (Spiegel et al. 2004). Other coprophilous fungi (e.g., ascomycetes, basidiomycetes) can be dried together with the dung and stored in paper bags or in cardboard boxes (Agarwal and Sharma 2006).

Further protocols for microfungi associated with plants and animals can be found in Mueller et al. (2004). Additionally, Crous et al. (2016) provide techniques for isolation, cultivation, and molecular and morphological studies of filamentous fungi and yeast.

Plant-Pathogens

Plant pathogenic fungi and oomycetes can be detected either by the appearance of disease symptoms or by mycelia/fruiting bodies growing on diseased or healthy plant tissue (Callan and Carris 2004). A description of symptoms and pathogens is found in Shivas et al. (2005) and Thines and Choi (2016), the latter focusing on biotrophic oomycetes. Species identification without the aid of sequence data is in most cases only possible after identifying the host plant. Both diseased and uninfected plant material should therefore be collected (Callan and Carris 2004). It is essential to heed the following recommendations (Callan and Carris 2004; Shivas et al. 2005; Buyck et al. 2010; Lane 2012; Prospero et al. 2017):

1. Do not collect in the rain, as wet samples may become colonised by other organisms.
2. The whole plant should be examined during fieldwork, starting from the youngest leaves, and working toward the stem. Ideally, samples should be taken from all symptomatic parts of the plant.
3. When sampling trees, the upper and lower side of an angled trunk or branch should be sampled, along with various strata (canopy,

trunk, ground). Epiphyte flora will be different due to different moisture conditions.
4. If the plant has a poor crown, the roots should be examined.
5. Collected samples should include different aspects and symptom stages of the disease.
6. Samples should include the border between healthy and diseased tissue.
7. Rotten material and dead plants should be avoided, as pathogens may no longer be viable and saprobic fungi may have already colonised these parts.
8. All sample tools should be disinfected after use with 70% alcohol or 5% bleach (NaOCl).
9. Avoid heating samples to more than 30 °C.

RECOMMENDATION
It is crucial to record sample details, symptoms, and symptom intensity. It is also convenient to use a disease assessment scale along with taking photographs in the field. A checklist of what to look for when examining plant samples can be found in Lane (2012) and Taylor (2018). Refer to Schubert et al. (1999) for further sampling considerations.

To sample the appropriate parts of the plant, some previous knowledge of disease symptoms is useful (Shivas et al. 2005). The most common parasitic fungi and oomycetes found in both agricultural and natural environments include rust fungi, which may produce various types of spore-producing structures that are often orange or brown in colour and can be found in different infection stages on leaves, shoots, fruits, and woody stems of a single host. Smut fungi can be found in specific parts of the host crop plant, especially the leaves, stem, roots, and inflorescences and often produce a mass of dark brown to black teliospores. Powdery mildews can be recognised by the white and dusty conidial state found on leaves and stem surfaces. Sooty fungi can form a hyphae network or a dark crust on leaves and small twigs (Callan and Carris 2004). Downy mildews cause chlorosis of infected leaf parts and often stunting in systemic infection, and produce a whitish, violet to dark brown down, predominantly on the lower leaf surfaces, as sporangiophores usually protrude from the host through the stomata (Thines and Choi 2016). White blister pathogens produce white to off-white pustules on infected plant parts that disseminate a white spore powder after pustules rupture (Heller and Thines 2009).

When collecting, leaves should be wrapped in moisture-absorbing paper and placed in paper bags to avoid desiccation (Shivas et al. 2005; Lane 2012). If the ambient air is too humid, a small quantity of desiccant can be added to the bag (Callan and Carris 2004). Alternatively, samples may be placed in a plastic box containing moist paper, covered with a lid, and stored in a cold room at 5 °C. They can be stored for up to 3–4 days. (APS 2022). If whole plants are sampled (i.e., small herbaceous plants), they should be placed in strong plastic bags with the roots tied off at the collar and stored in a cardboard box lined with absorbent paper (Lane 2012). Do not pull plants from the soil and scrubbing should be avoided when cleaning the roots (Shivas et al. 2005). Fruits, flowers, and tubers should be collected in the early or intermediate stages of the disease (Shivas et al. 2005), individually wrapped in dry paper and placed in open or perforated plastic bags, and should be carried in a robust container until desiccated or cultured (Lane 2012; Callan and Carris 2004). Conifers and spiny plants should be stored in large paper bags. Cankers on trunks should be removed with a knife or a chisel and should be wrapped in newspaper or in a paper bag (Callan and Carris 2004). Refer to Prospero et al. (2017) for detailed sampling protocols for bark, wood, shoots, leaves, and roots.

If the collected material cannot be cultured within a few days, samples can be air-dried after pressing them between blotter sheets. Specimens can be wrapped in newspaper for transportation and stored in paper packets (Wo et al. 2004; Buyck et al. 2010). Cultures can be obtained later if the samples contain immature fruiting bodies close to sporulation (Callan and Carris 2004). Rust and smuts can be frozen

at -20 °C for up to seven days after drying, for later examination (Shivas et al. 2005).

If fungal pathogens are present inside the plant tissue (e.g., leaves, petioles, roots, or branches), tissue should be washed with 70–95% ethanol, followed by immersion in 0.5–10% sodium hypochlorite (ideally with 0.1% Tween20 or a similar detergent), and rinsed with autoclaved distilled water to kill surface contaminants such as saprobic fungi (Schmit and Lodge 2005; Shivas et al. 2005; Beales 2012). Some previous experimentation should be conducted to ascertain the correct bleach solution and exposure time for specific plant or fungal associations, as some plant tissue may be too delicate, or some fungal structures may not be affected by this treatment (Lori M. Carris, pers. comm.). The outer layers of the tissue should be removed, and the inner part should be cut into small pieces (ca. 2 mm) and placed on Petri dishes containing an agar medium (Narayanasamy 2011). Adding acidified malt can restrain bacterial growth and stimulate pathogenic fungi growth instead (Callan and Carris 2004). This also applies to endophytic fungi that do not cause diseases.

Leaf washes is another sampling alternative that also works for spores of saprotrophic fungi (Schmit and Lodge 2005). Detailed protocols for isolating pathogenic fungi from leaves, stems and roots can be found in Shivas et al. (2005). Growth requirements for specific fungi are defined in Ryan et al. (2012).

Fungi and allies can be identified by examining them under a compound light microscope. However, if reproductive structures are not present, the interface between healthy and sick tissue (ca. 2-mm sections (Akinsanmi et al. 2004)), where the pathogen is most active, should be cultured as soon as possible. Do not use necrotic tissue, as this may contain already secondary colonisers. If fruiting bodies close to sporulation are seen, the substrate can be incubated in a moist chamber for one to three days to encourage sporulation (Callan and Carris 2004).

Several microfungi can be grown in pure culture except for those that are obligate parasites or biotrophs (i.e., downy mildews, rusts, black mildews, and powdery mildews). Few obligate species may grow *in vitro* under non-axenic conditions and only together with the living host (Callan and Carris 2004), making this technique very laborious and expensive (Ryan and Ellison 2003). Thus, drying is the best way to preserve obligate parasites (Callan and Carris 2004) and preserve them as herbarium specimens (Homolka 2014). Collection of rusts' basidiospores and teliospores is also possible following Ryan and Ellison (2003). However, the described method does not generate sufficient spores for preservation, even though molecular identification might be possible.

Leaf surface impressions or peels, adhesive tape, transparent cellophane tape, Mellinex, or dry water-based mucilage are additional methods to examine recalcitrant pathogenic microfungi structures (Callan and Carris 2004; Narayanasamy 2011). These procedures may be suitable for DNA recovery by touching a single lesion of a leaf so that spores can attach to the tape, which should be cut into small pieces and placed in small tubes (Pilo et al. 2022). Note that the sample will probably contain other fungi, bacteria, and PCR inhibitors (Lori M. Carris, pers. comm.). Hence, the material should be treated as an eDNA sample, unless species-specific markers are to be used.

Microbiomes

Environmental microbiomes are systems, found together in the same habitat, that include a variety of microorganisms such as bacteria, archaea, fungi, algae, and protists, together with mobile genetic elements such as viruses, phages, and relic DNA (Berg et al. 2020; Ryan et al. 2021). So far, targeted preservation of environmental microbiomes is very rare (Smith et al. 2020; see Steiger and Heuss 2020 for a list of existing projects), as culture collections are usually maintained under axenic conditions, and only freeze-tolerant organisms endure cryopreservation procedures, leading to a partial loss of the microbial community (Ryan

et al. 2019). Moreover, best practices and data standardisation in microbiome research is lacking (Berg et al. 2020). The **MicrobiomeSupport** project has been set up in 2018 and its main aim is to provide and improve standards and protocols for microbiome studies. Further details about concepts and challenges can be found in Berg et al. (2020).

Note that a microbiome sample will only represent the temporal and spatial structure of the moment when the sample was collected.

Sample collection and duration, as well as temperature during transportation and storage are crucial factors for maintaining the microbial community composition. Samples for intact microbiome preservation should be placed in the dark at temperatures lower than 4 °C directly after sampling. At the laboratory, samples should be immediately stored in a refrigerator (Prakash et al. 2020).

Currently ongoing studies will help to develop optimal preservation methodologies that can maintain the integrity, functionality, and inter-species interactions of microbiome samples (Smith et al. 2020; Ryan et al. 2021). Microbiome resources, such the **Microbiota Vault**, **the Alliance for Phytobiomes** and the Minimal Effective Microbiome Set (MEMS) for a plant microbiome vault are being established to provide repeatability in microbiome research (Gopal and Gupta 2019; Ryan et al. 2021). In addition, the **Earth HoloGenome** initiative is taking the lead on the standardisation of collection and preservation practices on animal-microbiota associations.

RECOMMENDATION
A description and documentation of the site location, habitat, topography, nature of the substrate, host identification (if present), and associated flora and fauna should be carried out and documented with photographs.

Macrofungi

Samples of macrofungi, the so-called "mushrooms" (mainly basidiomycetes and sometimes ascomycetes), should be in good condition, and not over-mature or too immature, decayed, dried, or damaged. They should not contain mites or insects and, ideally, should be fertile and contain spores (Shivas et al. 2005; Prance and Fechner 2017). For species identification, it is crucial to gather material at various stages of maturity (Lodge et al. 2004; Buyck et al. 2010). The whole fungal fruiting body should be collected, including ring, volva and sclerotium, as well as some of the basal mycelium, if present. Mushrooms and resupinated fungi should be sampled together with the substrate, using a small trowel or a truffle rake for digging up, or a pruning knife to cut tree bark. Fungi should never be pulled out, as some parts may get detached, and the sample will be less valuable (Lodge et al. 2004; Shivas et al. 2005; Buyck et al. 2010; Prance and Fechner 2017). Collect as much material as possible, as some of it may be destroyed during the identification process. Therefore, 20 small fruiting bodies, 5–10 medium-size fruiting bodies or one specimen for very large fungi is recommended (Prance and Fechner 2017), though the number of available individual fruiting bodies in the population should be considered.

Large robust specimens can be wrapped in paper bags, waxed paper, or aluminium foil. Smaller or fragile specimens can be placed in rigid containers packed with moss to maintain a high humidity for transportation (Buyk et al.

2010), or wrapped in aluminium foil (Lodge et al. 2004). Airtight containers should be avoided, as bacteria can quickly multiply within, damaging the specimens (Castellano et al. 2004). It is crucial to avoid contact between specimens from different collections as spore cross-contamination may occur. If field conditions are warm or if samples cannot be at the processing place within one hour of sampling, specimens should be kept cool and insulated in a cooler box to avoid rotting (Buyck et al. 2010; Prance and Fechner 2017). Drying should occur as soon as possible, both for the fungus and its substrate. Small fungi can be air-dried, while large, fleshy fungi should first be cut in half to ease drying, then air dried at 40–42 °C using a desiccator (Wu et al. 2004; Shivas et al. 2005); they can also be placed in an airtight container with silica gel to avoid deterioration (Castellano et al. 2004). Alternatively, mushrooms can be oven-dried at 70 °C for 3–4 h, without affecting DNA quality (Wang et al. 2017). When totally dried, samples will turn brittle, so they should be placed in paper bags or Ziploc bags for long-term storage (Wu et al. 2004; Prance and Fechner 2017). Macrofungi should never be frozen, as they will disintegrate when thawing (Kendrick 1969). Further sampling protocols can be found in Mueller et al. (2004) and O´Dell et al. (2004).

RECOMMENDATION
Spore prints can be taken. Follow Lodge et al. (2004), Buyck et al. (2010), and Prance and Fechner (2017) for details.

Fresh fungal tissue can also be stored in sterile vials containing 2× CTAB buffer or on FTA cards. Tissue from the gills that looks clean, or tissue from the inner cap or stipe, should be removed directly after collection for DNA analyses (Lodge et al. 2004; Buyck et al. 2010).

RECOMMENDATION
FTA cards are used for storing tissues from different organisms. Do not overload the cards with excessive amounts of tissue, because this would be detrimental for further sequence recovery. Do not expose cards to humidity.

Lichens

Whole fertile lichen thalli should be sampled, unless they are rare or uncommon (Will-Wolf et al. 2004). Foliose species are easy to collect following the bryophyte sampling protocol (refer to the plant section below). Crustose species should be sampled using a hammer and a chisel because they adhere strongly to rock or bark surfaces. It is recommended to dampen thalli before sampling to keep the specimens intact during collection (Buyck et al. 2010). Lichens can be air-dried in the sun and placed in packets made of wax paper or newspaper (Buyck et al. 2010). Brittle lichens should be dried together with their substrate. Leaflike and branched lichens can be flattened and pressed, whereas lichens attached to rocks should be wrapped separately in tissue paper when dried. (Wu et al. 2004). It is recommended to never place lichens in watertight containers, as mould can overgrow them (Will-Wolf et al. 2004). After drying, the lichens can be frozen at -20 °C to kill any arthropods. Identification of lichens usually requires microscopy and chromatography (Buyck et al. 2010).

For molecular genetic studies, fresh pieces of thalli or apothecia can be placed directly into Eppendorf tubes, 96-well plates or vials, where they can be left to dry, with open tube lids, in a drying chamber including silica gel (Marthinsen et al. 2019).

Benthic algae

After phytoplankton, periphytic and benthic are the most common algal growth forms, including both macroalgae and microalgae (e.g., filamentous algae) (Porter et al. 1993; Wade et al. 2020). Several macroalgae also occur unattached, both in freshwater and marine environments. Sampling will depend on the substrate (e.g., boulder, cobble, wood, sand, mosses) to which algae are attached. Scraping and brushing are generally performed for microalgae, using scalpels, spatulas, or brushes (BSI 2003; Francoeur et al. 2013; TCEQ 2014). Substrata can be carefully removed from the water for sampling or, if submerged, they can be enclosed with a Ziploc bag to avoid losing any dislodged algae. Ideally, algae should be sampled together with the substrate to maintain material integrity (Francoeur et al. 2013). Detailed sampling protocols for each substrate type can be found in TCEQ (2014) and Moulton II (2002). Metaphyton is found as aggregations or clumps between planktonic and benthic habitats and can be sampled either by gathering algae into a jar or a syringe, or by hand (Francoeur et al. 2013).

Macroalgae can be collected in offshore waters (subtidal zone) by snorkelling (at depths up to 3 m) or scuba diving (3–30 m), whereas intertidal algae are usually collected at low tide by walking along the shore (Coppejans et al. 2009; Neto et al. 2020). Sampling in deeper water (exceeding depths of 30 m) can be performed by dredging or with **submersibles**. The "hook" collection method can also be implemented for subtidal algae (Tyler 2000). Samples can be obtained using forceps, scalpels, or hammer and chisel, especially for crustose and mat-forming seaweeds, or by hand (Dhargalkar et al. 2004), and they should be placed in Ziploc bags containing a small amount of water to avoid dehydration (Porter et al. 1993; Johnstone et al. 2002; Fort et al. 2020). Large specimens can be collected in mesh bags (Barrento et al. 2016). Intertidal seaweeds can be wrapped in paper towels (Kawai et al. 2005). Note that, if possible, both sporophyte and vegetative tissue should be collected for further gametophyte isolation and genetic characterisation (Perrineau et al. 2020). Algae should be maintained in low-light conditions in an insulated container, at 4 °C for cool-temperate species, or at 18–20 °C for tropical taxa, for immediate processing (Kawai et al. 2005; TCEQ 2014; Smithsonian Institution 2020). If samples cannot be processed within 24 h (e.g., when sampling in remote locations), algae can be treated with 3% Benomyl fungicide to improve post-storage algal viability (Johnstone et al. 2002).

Samples should be rinsed with filtered or sterile seawater or distilled water on a tray and allowed to soak for one minute to remove sediment and release all epifauna adhered to them (Dhargalkar et al. 2004; Smithsonian Institution 2020). A paintbrush can also be used to help to clean algal tissue (Kawai et al. 2005). To release spores or gametes, right after collection, thalli should be immersed in freshwater for 10 min (Kawai et al. 2005; Bhattarai et al. 2007; Zhang et al. 2007). Algae can be transferred to -20 °C for further molecular and morphological analyses (Prasanthi et al. 2020).

RECOMMENDATION
If it is not possible to identify algal specimens to the species level, the functional group should at least be determined (Smithsonian Institution 2020). Taxonomic keys are available from many countries and regions around the world. A good electronic resource to get started is **AlgaeBase**.

Plants

During botanical expeditions, be aware of poisonous species, or plants with stinging hairs, thorns, or prickles especially if you are not familiar with the regional flora (Zippel et al. 2010). Plant material can also be obtained from living plant collections (botanical gardens, arboreta, and field banks), which are valuable sources for rare taxa that cannot be easily collected in the field. For material from living plant collections, a photograph of the plant label should be taken (Gostel et al. 2016). Tissue can be stored directly in LN2 tanks, as the living plant collections usually have their own repository or facilities nearby. A workflow standard for sampling and handling plant tissue in botanical gardens has been developed by Gostel et al. (2016).

RECOMMENDATION
Collection of material for genetic analyses should be a common practice during botanical field expeditions. All plant tissues must be linked to their respective herbarium voucher to allow confirmation of taxonomic identification.

Living vegetative tissue sampling collection

If living material is required (e.g., propagation), a field tissue culture can be initiated by *in vitro* collecting (IVC). It is important to note that this procedure has to be adapted to the specific taxon and material to be used (Sarasan 2010). IVC is suitable for species that do not produce seeds, when seeds are not available at the time of collection, or for species with recalcitrant seeds (see definition below). This additionally allows avoiding problems associated with transporting large amounts of vegetative tissue, which can die before reaching the biobank (Pence et al. 2002). However, contamination remains a large challenge, as work is performed in the field and cultures may be exposed to both air-borne contaminants and endogenous microorganisms (Cruz-Cruz et al. 2013). Check Pence et al. (2002) for contamination control protocols during IVC.

Several factors are, therefore, critical and should be considered for a successful outcome: 1) type and size of tissue, 2) removal of soil remnants and pests, 3) avoidance of damaged tissue, 4) tissue sterilisation and washing, 5) nutrient media with antimicrobial additives and, 6) storage conditions (Cruz-Cruz et al. 2013).

The material to be collected depends on the species. In general, budwoods, shoots, apices, or leaves can be used to initiate IVC. It is necessary to collect stakes, pieces of budwood, tubers, or corms for vegetatively propagated species (Cruz-Cruz et al. 2013). Two methods can be applied for tissue collecting (Pence 2005):

- *The leaf punch method*. Leaf discs (ca. 6 mm) are punched from young leaves and placed into vials with media including fungicide and antibiotics.
- *The needle collecting method*. A cross-section from soft, green, young stem tissues is collected using a syringe with a gauge needle (no. 21) to obtain a tissue cylinder which is placed into a vial with medium. Very small stems can be sliced using scissors.

Collected material can also be stored in Ziploc bags containing moist paper towels and processed indoors within a few hours of collecting if outdoor processing is not performed (Pence 2005). For culture conditions, refer to Pence (2005). Vials should be carried in opaque cloth bags and direct sunlight should be avoided. Samples can be left at ambient temperature in the field (Pence 2005). Protocols for various crops, tropical rainforest trees and endangered species can be found in Pence et al. (2002).

Collection of cuttings is preferable over tissue sampling for succulents and woody species propagation. Cuttings should be clean and

neither too woody nor too soft. Cuts should be made just above a node or bud from one plant using secateurs. Secateurs or pruners should be decontaminated with 70% alcohol between plant collections. All cuttings should be free of flowers, flower buds, and fruits to minimise moisture loss. Cuttings should be stored moist and cool (3–5 °C), loosely wrapped in newspaper and placed into plastic bags. Cuttings that have been wrapped for more than 24 h should be exposed to release ethylene, which can lead to tissue damage after removing the material from the parent plant (**Australian National Herbarium**, updated 2015; Martyn Yenson et al. 2021). Types of cuttings, preparation and growing conditions for cuttings are available in Martyn Yenson et al. (2021).

Plant material collection for DNA banking and germplasm conservation

The selected tissue preservation method will depend on the species and the duration of the field trip. Preservation methods include the use of RNAlater or equivalents, desiccation with salt buffers (e.g., NaCl-CTAB buffer), 96% ethanol (Bressan et al. 2014; Johnson 2021) or silica gel, which is the most common plant material preservation method used during fieldwork (Chase and Hills 1991).

Silica gel should ideally have a 28–200 mesh size grade and include a proportion of beads that contain a moisture indicator dye that changes from orange to colourless as gel saturation levels increase (Chase and Hills 1991). Blue dye indicator should be avoided, as it contains cobalt chloride, which is carcinogenic (Funk et al. 2017; Maurin et al. 2017). Relative humidity indicator cards provide an alternative to indicating gel (Funk et al. 2017).

RECOMMENDATION
All grades of silica gel can cause respiratory problems if inhaled and can also be a dermatological irritant. Use a facemask if working with finer grades of gel, and wear gloves if working with silica gel over substantial time periods. If possible, rinse any powder off the outside surfaces of tubs and bags when you have finished working with them.

Placing samples straight into Ziploc bags containing silica gel makes the replacement of saturated gel with dry gel, and any reuse of the silica gel, time consuming due to the physical contact between the plant material and the gel (Wilkie et al. 2013; Funk et al. 2017). Instead, the plant tissue can be placed in porous paper coin envelopes or tea bags/coffee filters, sealed by folding over the top and pinning with archival paper clips to ensure that the plant material does not escape (Wilkie et al. 2013; Maurin, et al. 2017). Such sample bags can be placed either into Ziploc bags or into an airtight plastic container filled with silica gel for drying. The amount of silica gel will depend on the tissue water content, but 10–15 times as much silica gel as tissue should generally be used (Chase and Hills 1991). Although coin envelopes are not usually archival quality paper, and tea bags are less robust, they can both be used for long term-storage, minimising laborious post-collecting processing work (Gemeinholzer et al. 2010; Funk et al. 2017, Maurin et al. 2017). Samples should be kept in a cool, ambient-temperature environment, and the silica gel should be changed within 12 h if the tissue is not completely dry (Davis 2011). Ziploc bags and plastic containers have to remain closed with an airtight seal to ensure that the silica gel maintains its desiccating properties (Maurin et al. 2017). Once the material is completely dried (after 24–48 h), the envelopes/tea bags/filters can be transferred into individual Ziploc bags with a small amount of fresh silica gel (Funk et al. 2017; Maurin et al. 2017; Duque-Thüs and Fulcher 2018), kept in plastic lunchboxes, or stored in low humidity cabinets (Plaisier 2019: **Botanics stories**). Samples can be stored at -20 °C or -80 °C (Funk et al. 2017), or if no sub-zero facilities are available, they can be kept at room temperature, if stored in hermetically sealed containers (Hodkinson et al. 2007; Tim Fulcher, pers. comm.).

Another alternative is to use FTA filter cards to preserve leaf material. Leaves should be carefully crushed onto the card, avoiding cross-contamination. Between 50–100 µl of plant homogenate can fit on one card. Cards should be placed into multi-barrier pouches containing desiccant packets, to ensure that cards remain dry during storage and transportation (Gemeinholzer et al. 2010).

For a comprehensive publication on plant tissue collection for DNA banking, see Funk et al. (2017). This paper provides recommendations for all the tasks required for voucher preparation, sample collection, LN2 preservation, and storage. The Queensland Herbarium (2016) provides collecting protocols especially for difficult plant groups such as bamboos, palms, and cacti. Barber and Galloway (2014) compiled methods for the collection of seeds, fruits, spores, underground organs, and cuttings and their further processing during fieldwork.

The following section provides guidance on collecting in the field for each plant group.

Bryophytes

Mosses, hornworts, and liverworts can be collected by hand, with a knife if they are firmly attached to the substrate, or with masking tape for tiny bryophytes (Glime 2017). For field preservation they can be placed into folded paper envelopes or paper bags, with smaller specimens or delicate structures such as sporophytes first being wrapped in mini-packets to prevent loss or damage. Very damp specimens can be gently squeezed to remove most of the free water and placed in double envelopes. Ground-dwelling species can be stored into small boxes to avoid the plants from fragmenting and mixing with soil particles (Vanderpoorten et al. 2010). See Glime (2017) for more detailed methods and Hart and Forrest (2020).

Prior to DNA preservation, living bryophyte specimens should be carefully cleaned, and insects, contaminating plant fragments and soil should be removed using forceps and water under a dissecting microscope. This is most easily done before the bryophyte specimen is totally dry; rewetting a dried sample during cleaning should be avoided or minimised as this can damage the DNA. Scales and rhizoids of thalloid liverworts can also be removed, using forceps or a razor blade, as they can trap debris and rhizoids tend to contain fungal endophytes (Forrest et al. unpublished). After cleaning, dry the samples as soon as possible to avoid DNA degradation. A hot air-drying (40–80 °C) method, using either a portable hair dryer, fan heater or electric blanket, has been suggested instead of the more commonly used silica gel method, and may be beneficial as samples in silica gel are usually brittle and easily break into tiny fragments, which are hard to gather for DNA extraction (Zhao et al. 2019; Forrest et al. unpublished). Other laboratories store a carefully cleaned subset of each bryophyte collection in Ziploc bags containing silica gel for DNA extraction, while the rest of the collection is stored in the paper envelope and forms the herbarium voucher.

RECOMMENDATION
Do not reuse silica gel since the risk of cross-contamination is high due to small plant remnants. In addition, take care if storing bryophytes in rough paper teabags, as they can stick inside the bag.

If fieldwork does not allow for on-site sample cleaning and processing, collected bryophytes may be placed into Ziploc bags or plastic lunchboxes, where they can survive for over a week. Material should be stored in cool boxes to restrict fungal growth. Back in the laboratory, it is possible to clean living samples externally using sonication and bleach techniques, noting that plants are likely to lose their colour; however, this level of sterilisation is not generally required for routine sample preservation.

Be aware that collected bryophyte clumps very commonly include more than one species, and it is easiest to subsample and identify these while the material is still alive and flexible enough to untangle without breaking, and while important morphological characters like oil bodies are still present. Several healthy

similar stems or thalli are usually sampled for routine DNA extraction for Sanger sequencing (e.g., DNA barcoding). However, samples of multiple stems from a clump may include tissue from multiple individuals, leading to multiple genotypes in one single DNA extraction. If it is necessary to avoid this (e.g., for genomic analysis), take care that all the material for DNA extraction is physically connected and so clearly part of the same individual, e.g., only sample a single stem per vial, and take care to avoid dwarf males or sporophytes (Forrest et al. unpublished).

RECOMMENDATION
Avoid rewetted material that will have undergone additional drying processes for DNA extraction, as its DNA is likely to get degraded.

Ferns and lycophytes

Sporophyte shoots and fronds, rhizomes, mature spores, and gametophytes can be collected into vials containing NaCl-CTAB buffer (Prendini et al. 2002) for DNA preservation. Apparently, the addition of sodium ascorbate acting as an antioxidant improves DNA extraction (Thomson 2002). Silica gel can also be used (e.g., Zhou et al. 2015; Wang et al. 2016), but DNA extraction has resulted in lower DNA yields (Neves and Forrest 2011). Although gametophytes are small and may be very difficult to identify, they are preferred for genomic studies, as ferns are at the larger end of the plant genome size spectrum (Laura Forrest, pers. comm.).

Spores. Spores are used exclusively for propagation and *ex situ* conservation purposes rather than for molecular analyses. Spores are collected from whole fronds or some pinnae containing completely formed sori. Fronds should be sampled just before the sporangia open, and they should be placed on sheets of glossy or plain paper to dry for up to a week at ambient conditions (between 30–60% Relative Humidity (RH)) (Nebot et al. 2021). Within that time frame sporangia will open, and spores will be released (Ibars and Estrelles 2015). Fern fertile leaves (e.g., *Matteuccia*) should be immersed in water for some hours to trigger leaf opening before drying. (Ballesteros and Pence 2018). Spores should be sifted with a sieve (50–150 µm) to remove remaining sporangia and paleae tissues (Ballesteros et al. 2012; Ibars and Estrelles 2015; Nebot et al. 2021). Spores can be dried for 2–7 days in a controlled environment using saturated salt solutions (e.g., $MgCl_2$, RH chamber, 30–40% RH, 5–20 °C). Silica gel may be used if RH is higher than 60% for up to two days (Ballesteros and Pence 2018), otherwise it should be avoided, as silica gel provides very low RH (usually <15%), which can be deleterious for the spores (Daniel Ballesteros, pers. comm.). If insufficient spores are collected (less than 70% of the vial), sporangia and frond tissues can also be stored together with the spores.

RECOMMENDATION
Ideally mature spores should be collected for long-term storage. Mature sporangia are bright in colour, either dark green for green spores, or brown, yellow, or orange for nongreen spores. The colour of the indusia will also help to recognise mature sporangia. If the sporangia are already opened, remaining spores can be collected and germinated in vitro to grow the gametophytes either to immediately cryopreserve them, or to collect spores under more controlled conditions (Ballesteros and Pence 2018).

Herbarium specimens have also been used to retrieve viable spores, which may have the potential to propagate and recover threatened species (see Ibars and Estrelles 2012). For further information regarding herbarium material, refer to the ancient DNA section.

Seed plants

Fresh mature leaves are the most frequently used plant tissue for DNA analyses. Three to

five leaves (depending on leaf size), equating to an area of 5–10 cm^2 or a weight of 2–6 g, are usually sufficient to collect during fieldwork (Davis 2011; Maurin et al. 2017). Avoid leaves that are damaged by insects, show signs of necropsy or sickness, or are colonised by lichens or moulds. Other tissues, such as leaf buds, petals (especially for orchids (Fay et al. 2006) and begonias (Laura Forrest, pers. comm.)), and bracts can also be sampled. If bark is the only accessible tissue (e.g., trees with a high canopy), the cambium beneath the bark should be scraped into a collecting bag/tube, whereas only flexible twigs with the soft pith inside should be sampled (CSH 2018). If tissue material looks dirty or is visibly contaminated with insects, fungi, or epiphytes, it can be cleaned with an ethanol wipe. To ensure proper preservation, pine needles, large leaves and leathery and waxy leaves should be cut into small pieces (ca. 2 cm in diameter) using scissors, and thick midribs should be removed. Avoid tearing, which may cause leaf bruising, and the release of enzymes that can break down DNA. Parenchymatous tissue can be removed from succulent leaves using a clean razor blade, keeping only the epidermal slices (Maurin et al. 2017). Fresh cactus spines (5–15/individual) from growing stem apices can also be collected by clipping them at their base (Fehlberg et al. 2013). Dense hairs should also be removed from the surfaces of pruinose or otherwise overly hirsute leaves (Gemeinholzer et al. 2010) by scraping or use of sticky tape.

Aquatic plants are more delicate than terrestrial plants and should be treated with care. Usually, a medium sized hoe is enough to collect samples from shallow waters, whereas rigid hooks with weights attached to a rope are required to sample in deep waters. Small water plants are frequently collected as a whole, whereas one or two leaves are enough for large water plants (Tomović et al. 2001). Plants should be placed on cards, which in turn are placed between newspaper sheets. Newspapers should be changed several times until specimens are totally dry (Valdecasas et al. 2010).

RECOMMENDATION

Try to avoid sampling roots, which may contain mycorrhizal fungi or rhizobia, or thorns and spines, which do not usually contain a lot of DNA. Tubers are also a poor sampling choice due to high levels of starch. Bud scales contain lignin, which hampers extraction, if possible, they can be removed from the buds with forceps. Sepals, petals, and fruits may contain secondary compounds that can be problematic for DNA purification, resulting in lower yields of DNA.

Tissues have to be dried using silica gel directly after collection for subsequent preservation, storage, and DNA extraction (Hodkinson et al. 2007; Gaudeul and Rouhan 2012). Tissue from succulents or other notoriously hard to extract plants (e.g., xerophytic plants) can also be preserved in a high salt buffer, (e.g., NaCl-CTAB buffer) or RNAlater. It is highly recommended that such samples should be processed as soon as possible after returning to the laboratory, as room temperature liquid preservation is not suitable for long-term storage (Prendini et al. 2002; Hodkinson et al. 2007; Gemeinholzer et al. 2010). Testing over the course of a year has shown that the buffer-preserved tissue should be stored at low temperatures; DNA extractions from buffer-preserved samples stored at -20 °C are comparable to those from silica-gel preserved samples (Hollands 2018: **Botanics stories**). Removing excess buffer from the tubes prior to freezing makes subsequent sampling more straightforward (Funk et al. 2017).

When used on plants, the alcohol or Schweinfurth method appears to damage DNA, making it difficult to extract long enough fragments for PCR amplification (Staats et al. 2011; Särkinen et al. 2012; Forrest et al. 2019).

If fresh tissue is required and transport duration is no longer than three days, it may be possible to prevent deterioration by keeping tissue cool and moist (Funk et al. 2017). Plant tissue should be placed into Ziploc bags with damp (not wet) paper towels but note that this may promote rotting of the material (Prendini

et al. 2002). The bags of tissue are then stored in a styrofoam box containing ice. If dry ice is required, place the samples on newspaper to avoid cold temperature burns (Prendini et al. 2002). Succulent and xerophytic plant material can also be kept at room temperature in Ziploc bags for up to a day, without damaging the DNA (Neves and Forrest 2011).

Follow Spooner and Ruess (2014) for detailed tissue preservation and storage methods.

Pollen banking

Pollen banking has had a limited use in plant germplasm conservation, but it may be a valuable method for species with recalcitrant seeds, and for different horticultural plant species (Theilade and Petri 2003). Most methods described below have been mainly developed for crops and commercial species.

RECOMMENDATION
It is essential to know the cellular trait, as it is the foundation for establishing pollen viability tests and storage conditions. Bicellular pollen is usually desiccation-tolerant, longer-lived, shed at a lower moisture content and can be germinated in vitro. In contrast, tricellular pollen (found in Graminaeae, Umbelliferae, Cruciferae, Araceae, Caryophyllaceae and Chenopodiaceae) typically has a high moisture content, endures a limited desiccation, and viability is measured in situ in styles or seed set (Towill and Walters 2000). A DAPI staining technique can be used to elucidate the pollen nature.

Mature pollen collection must occur in the morning soon after flowers are fully opened (anthesis) (Prendini et al. 2002) by shaking the flowers over a sheet of butter paper or by using a sieve. Usually, anthers are collected when species do not shed vast amounts of pollen. Pollen should not be collected from sick or damaged flowers. Both pollen and anthers should be processed soon after collection to guarantee maximum potential longevity (Volk 2011). Be aware that large amounts of pollen are required not only for storage, but also for moisture measurements, viability testing and subsequent breeding programs and crop improvement.

Depending on the species, pollen has to be separated from anthers prior to desiccation. Otherwise, the whole anther can be dried and gently crushed (Towill and Walters 2000). Sticky pollen should be treated with *Lycopodium* powder or talc to avoid clump formation (Sidhu 2019). Samples can be air-dried on clean Petri dishes if the ambient humidity is low (Anushma et al. 2018). Otherwise, they should be desiccated using LiCl, $MgCl_2$, NaOH, or silica gel to reduce moisture below the species-critical level (Towill and Walters 2000; Sidhu 2019). Most pollen can be dried to less than 5% moisture content (Theilade and Petri 2003). However, some desiccation-sensitive pollen requires controlled conditions for drying and cooling, as well as the use of cryoprotectants for successful cryopreservation (Nebot et al. 2021).

Seed banking

Seed banking is the most widely used *ex situ* conservation method for crops and wild plants. In general, standardised protocols exist for the storage and preservation of crop species, but not specific ones for wild species, because the amount of collected seeds may limit the number of seeds available for testing protocols (Hay and Probert 2013). Seed banks should ideally have a base collection for long-term conservation, which should never be distributed for use, and an active collection for propagation, multiplication, and distribution (Cromarty et al. 1990; Engels and Visser 2003).

All following described procedures are dependent on the type of seed. Seeds are classified into three groups depending on their storage conditions:

- *Orthodox seeds* can be dried and stored at low temperatures without losing their viability; and, importantly, their longevity

in storage is increased in predictable ways by reductions in moisture content and temperature (Roberts 1973). Most crops produce this type of seed.

- *Intermediate seeds* cannot survive when exposed to both low humidity and low temperatures simultaneously (Ballesteros 2011). They are often chill-sensitive but can be desiccated until a certain threshold (Theilade and Petri 2003). Citrus, coffee, and rubber are economically important examples of species belonging to this category.
- *Recalcitrant seeds* are shed at high moisture content and do not tolerate drying. Most species bearing recalcitrant seeds are woody; and it is predicted that most tropical tree species bear this type of seed. Likewise, they do not have dormancy, or are resistant to dormancy induction procedures. They usually do not remain viable for long after falling from the tree if germination does not occur. (e.g., Wyse and Dickie 2018).

RECOMMENDATION
Before collecting, a pre-assessment of the population should be carried out either by visiting the site, checking herbarium data, or contacting local experts. It is important to identify species accurately, as well as to determine their fruiting period and seed dispersal timing. Refer to the **MSBP technical information sheet No. 2** for further information.

Martyn Yenson et al. (2021) have developed guidelines for the maintenance of seed collections, including identification of seeds, seed germination, dormancy, and plant nursery.

The physical quality of the seeds should be assessed, prior to collection, by using the cut-test technique. Seeds are cut along both axes to check whether they are infested, immature or empty (**MSBP technical information sheet No. 4**). If the proportion of damaged seeds is higher than 30%, an increased number of seeds should be collected to compensate for the non-viable ones or another population should be sampled (**MSBP technical information sheet No. 2**). Ideally, between 3000–5000 seeds from > 50 mother plants per accession should be randomly collected to guarantee that there is enough material for germination assays, long-term conservation, and propagation (CPC 2019). Obtaining this number of seeds for tropical species and many tree species is generally not possible. However, depending on the aim of the collection, seeds can be pooled across the population rather than keeping them separately per mother plant (Filip Vandelook, pers. comm.). No more than 20% of the seeds found in the population should be collected to avoid compromising its *in-situ* viability (Gold et al. 2004; CPC 2019).

RECOMMENDATION
For choosing the most convenient desiccation and preservation procedure, learn to understand the physiology of the studied species before going to the field.

Seeds have to be collected at the time of optimum ripeness, as this is a precondition to secure high seed quality (Schmidt 2000). Unripe seeds have a high RH, which may cause reduced storability and vigour (Theilade and Petri 2003), whereas seeds that have imbibed moisture and initiated germination will not tolerate re-drying (Schmidt 2000).

Many methods for seed collection have been developed, and nearly all of them can be equally applied to shrubs, herbaceous species, and trees. The choice will depend on the kind of plant to be sampled and the location. Ground collection is the easiest and most common way to obtain large seeds. However, the following issues should be considered when choosing this method: 1) the mother plant may be difficult to confirm, or a seed mixture is involved, especially in high-density stands, 2) seeds may be infested, 3) seeds can get lost on the ground, 4) seeds may germinate or deteriorate right after falling, 5) seeds from closely related species may be also sampled and, 6) debris, damaged seeds and soil-borne

pathogens may be collected when raking the ground or using mechanical equipment (e.g., vacuums, rotating brushes) (Schmidt 2011). An alternative for collecting small seeds is using tarpaulins or sheet funnels under tree crowns. Seeds from grasses can be collected by stripping entire seed-heads (**MSBP technical information sheet No. 3**). Explosive fruits can be collected by securing cloth bags over the seed head or fruit. Flowers from aquatic plants can be wrapped in nets to capture future fruits (ENSCONET 2009). Taller trees with seed-bearing branches can be reached by using long-handled tools (e.g., pruners, telescopic poles) or by shaking. Climbing using spurs, braided ropes, or tree bicycles is another alternative, but these techniques should only be used by trained people (Schmidt 2000).

Large fruits/seeds should be placed into clean baskets or buckets, non-glossy paper bags, or woven hessian sacks. Small dry fruits/seeds should be placed on canvas sheets that can be folded and tied (Schmidt 2000). Fleshy fruits can be placed into open plastic bags for a short period of time. Do not use large containers, as ventilation cannot be assured. All container types should be tag-labelled inside and outside. A compilation of seed collection details and procedures can be found in Schmidt (2000), ENSCONET (2009), and SOS (2018).

RECOMMENDATION
Seed sampling should not involve over-collection of the studied population, as it will put natural regeneration at risk.

If space is limited or field duration is long, a manual pre-cleaning from debris and plant remnants can be carried out. If the outside RH—determined by a hygrometer—is greater than 40–50%, plastic boxes containing small amounts of silica gel, charcoal or dried rice can be used for partial drying of orthodox seeds (ENSCONET 2009; Hay and Probert 2013). Otherwise, seeds can be placed in empty cloth bags for pre-drying (Engels and Visser 2003). Recalcitrant seeds lose water gradually; therefore, it is crucial to maintain the water level content at shedding levels (FAO 2014) by, for instance, storing them in the dark in closed bags to avoid drying (Ballesteros et al. 2021). Note that RH rises as temperature falls, especially overnight. Avoid extreme temperatures because they can damage the collection. Orthodox seeds can be exposed to direct sunlight after moisture reduction, whereas recalcitrant seeds should never be sun-dried (Schmidt 2000). Seeds should be stored in a cool dry location.

Seed extraction procedures are dependent on fruit and seed type. Seeds can be extracted from fruits (e.g., by drying, sifting, shaking, or flailing) in the field, unless high temperatures or threshing are required. Fruit pulp should also be removed, otherwise it will ferment, heat will be produced, and seed viability will thus be reduced (Schmidt 2000). Fruit pulp is sometimes removed upon returning to the laboratory, but it should remain fresh, as dry pulp is harder to eliminate (Filip Vandelook, pers. comm.). Some seeds cannot be separated from the fruit (e.g., nuts), as this will cause damage to the seed (Schmidt 2011). Follow Schmidt (2000) for seed extraction, depulping procedures, and temporary storage. A summary of post-harvesting recommendations can be found in the **MSBP technical information sheet No. 4**.

During transportation, cloth sacks containing orthodox seeds should be placed into cardboard boxes and covered with waterproof materials to protect them from moisture. Ideally, recalcitrant seeds should be stored in sacks/paper bags containing sawdust to prevent dehydration (Schmidt 2000; Schmidt 2011; **MSBP technical information sheet No.3**).

RECOMMENDATION
Seed deterioration depends on the species, the seed condition at collection and the environment (Schmidt 2000). Seed processing should be rapid, as it is crucial to maintain seed quality and viability. Ideally, the time between field collection and initial laboratory storage should be no more than five days (FAO 2014).

At the biobank facilities, seeds should be manually cleaned thoroughly or by using sieves of different sizes, rubber bungs, or aspirators to remove further debris under an extraction hood containing dust filters (ENSCONET 2009). For detailed cleaning procedures, see the **MSBP technical information sheet No. 14**. Seeds should be subsequently inspected for damage with a stereomicroscope and by using the cut-test (see above), and purity should be assessed using X-rays (MSBP 2015). Seeds that are empty, poorly developed, or insect-infested have to be discarded (ENSCONET 2009). If more than 50% of the tested seeds are hollow, this implies low viability, and another collection should therefore be considered (CPC 2019).

Collected seeds should be dried in a climate chamber, or in a desiccator containing either silica gel or salt solutions (Engels and Visser 2003). Regardless of the desiccation method, moisture content has to be regularly controlled, or seeds will degrade (Zimkus and Ford 2014). If immature seeds were also collected, they should be ripened before drying (MSBP 2019). Moisture content of orthodox seeds should be reduced as much as possible, and at least to the lowest possible level for recalcitrant seeds, which varies between species and within species, to avoid germination (Schmidt 2000, 2011). Consult the "culture preservation and storage" chapter of this handbook for further details on seed storage. In general, the drying process of orthodox seeds will take between one and four weeks, depending on the drying temperature and the RH. If drying at 25 °C, RH should be ca. 25–35%, whereas RH should be between 10% and 25% when drying at 5 °C (CPC 2019).

RECOMMENDATION
Never freeze seeds if they are not fully dried.

Immediately after drying and inspection, orthodox seeds should be packed in moisture-proof containers such as vacuum-sealed laminated aluminium foil bags, or air-tight containers (e.g., Kilner glasses) (Engels and Visser 2003; FAO 2014). Seed banking should occur within six months of collection or within two weeks after drying for short-lived and microscopic seeds (e.g., orchids) (MSBP 2019).

RECOMMENDATION
No chemical treatment of the material to control pests and diseases should be carried out in base collections. However, disease indexing should be performed, especially in vegetative form samples.

Animals

Livestock

Livestock species play an important role in food production and agriculture. Following domestication, selection based on human requirements led to the development of breeds, bloodlines and landraces carrying distinct genetic profiles. The term 'breed' also describes the unit of conservation that will be considered for conservation management plans (CGRFA 2012).

Biobanking of livestock resources helps to optimise management decisions, breeding strategies, development of new crosses and breed-types, genetic rescue translocations, and food security (FAO 2012). However, maintaining an updated collection is a crucial key point of livestock biobanking, as genetic differences between the *ex situ* collection and the *in situ* breeding population will emerge over time (FAO 2012; Blackburn 2018). Furthermore, populations from the same breed from differ-

ent geographical areas should also be sampled to uphold most of the genetic variability (Blackburn 2018). Samples must come from animals that are as unrelated as possible, as they will eventually be used for breeding purposes.

Collection and processing procedures depend on the sample type and the species. Field collection should follow strict protocols and staff should be trained for off-site collection, as disease transmission can occur when visiting different farms (FAO 2012). Consider the conservation and risk status of the breeds before sampling.

The FAO (2012) and the former IMAGE project (2020) have developed the following rules that should be applied during field collection:

- Disease testing should be accomplished before and after sampling collection.
- Ideally, samples should be collected at approved collection centres. If not the case, the sanitary status of the farm/herd/donor should be recorded as completely as possible.
- Samples from different species and with different sanitary statuses have to be placed in separate dry shippers, but may be stored in the same room, providing the tanks are clearly marked and no cooling agent can pass from one tank to the other.
- To minimise disease transmission, supplies, shipping boxes or any other instrument should not be used at other sites. These can be reused after cleaning and disinfection, but only at the same collection site.
- LN2 tanks or dry shippers used in the field should be warmed to room temperature and then decontaminated with a 10% bleach solution.
- Vehicle tires and undercarriage as well as boots have to be washed and disinfected after each farm visit. Boot covers can also be used and changed after each visit. Clothing should also be changed between collection sites or disposable suits should be worn instead. Polyvinyl or nitrile gloves should be worn and changed after handling each animal.
- Backup samples of non-germplasm material (e.g., blood) should also be collected.

RECOMMENDATION
Animal health principles and biosecurity must always be followed to avoid disease transmission. Quarantines may be required, as samples will originate from different animals, different farms, and possibly different countries.

Sperm collection. Semen collection is a common practice in several countries. Generally, collection procedures in mammals include electro-ejaculation, gloved-hand techniques, and the use of an artificial vagina, teaser female or a decoy animal. Abdominal stroking is used in poultry (FAO 2012). An inner liner should be placed in a collection bottle to trap the semen. For stallions, an extra non-woven filter pouch should be used to prevent the gel fraction from encountering the first sperm-rich fractions. Samples should be free of urine and other contaminants (USDA 2020). After collection, samples should be transported to the laboratory under a temperature/light-controlled environment, diluted and cryopreserved no later than 24 h upon arrival. Morphology and motility should be assessed before freezing. Species-specific collection protocols are found in FAO (2012) and USDA and can be adapted for field conditions.

When animals cannot be trained to undergo this procedure or are kept free range, it may be difficult to obtain sperm. An epididymal sperm post-mortem collection should be considered instead (ERFP 2003; FAO 2012). Preservation of epididymal sperm harvested from rats and livestock can be found in James (2004). Note that this type of sample will only preserve a single complement of chromosomes and paternal mitochondrial DNA will be lost, after fertilisation (Wolff and Gemmell 2008).

Oocyte collection. The most common method to collect oocytes involves removing ovaries from slaughtered donors. Ovaries should be kept warm in Ziploc bags during transport to the laboratory. If maintained cool, the *in vitro* fertilisation (IVF) embryo production rate will decrease dramatically (FAO 2012). Oocytes should be aspirated following the method described in FAO (2012, appendix H). Oocyte

quality should be assessed before oocyte *in vitro* maturation and IVF (FAO 2012, appendix I, J). Although these methods are described for cattle, they can also be applied to other livestock species (**USDA**).

Oocytes can also be harvested by implementing surgical procedures such laparotomy, endoscopy, and the transvaginal ultrasound-guided method (TUGA) (FAO 2012). The latter one is commonly used in cows, goats, mares, sow, llamas, deer, and antelope. For detailed methodology, see FAO (2012). Ovariohysterectomy has also been conducted in cats to obtain oocytes (Hobbs et al. 2012).

Note that freezing and thawing techniques are not as developed as those used for sperm, and further research is needed in this field (ERFP 2003). For instance, avian and fish oocyte cryopreservation has not been successful, due to their high lipid content and polar organisation (vegetal and animal pole). The alternative option would be to use fresh oocytes and frozen/fresh semen and freeze the resulting embryos instead (FAO 2012).

Embryo collection. Embryos allow the recovery of entire genomes without back-crossing. The first step to obtain embryos is to induce superovulation in donor females by injecting hormone agents. Subsequently, *in vivo* fertilisation takes place. A body flush procedure should be performed to make the embryos flow out of the uterus using a physiological flushing medium. Laparoscopic surgery is required in pigs, sheep, and goats. Ultrasonography should be used before embryo collection to evaluate the potential number of embryos that can be found. As soon as the embryos are collected, they should be placed into a hypertonic solution containing a cryoprotective agent, and cryopreserved. Freezing should take place when the embryos are at blastocyst stage, which is reached by five to nine days after fertilisation, depending on the species. Follow the FAO (2012) protocols for precise methods and recommendations.

Gonadic tissues / whole ovaries collection. Ovaries are obtained by laparoscopy or immediately after the donor female dies (**USDA**). The ovarian tissue is mainly used to restore an animal´s fertility by transplanting it into a recipient animal. The offspring of the recipient female will then carry the donor´s genotype. This method requires surgical expertise and special grafting facilities (FAO 2012).

Although avian oocyte cryopreservation is problematic, it is still possible to freeze ovaries from newly hatched chicks within the first 24 h, because at this age, their structure is different from that of the adult ovaries. Grafting of this tissue is feasible into one-day-old chick recipients (IMAGE 2020).

Primordial germ cells (PGC) collection. PGCs are embryonic diploid stem cells that are early precursors of gametes. So far, they have only been successfully reported in fish and birds. In chickens, PGC can be collected from embryonic blood, as the PGCs migrate to the developing gonads between the fourth and sixth day of incubation. PGC can then be propagated *in vitro* to increase their number (Blackburn 2006; FAO 2012; IMAGE 2020). A protocol can be found in **USDA**.

Somatic cell collection. A piece of tissue (e.g., whole or part of an ear) should be collected and immediately frozen to obtain somatic cells that can eventually be used for cloning. This technology has been used in many domestic species, but it requires a complex approach, including reprogramming the nuclei, enucleation of the oocytes, transfer of the somatic nucleus to an enucleated oocyte, culture of the resulting embryos, and transfer into recipients of the same species (FAO 2012).

Blood or serum collection. Blood is usually taken for veterinary diagnosis and evaluation from both living and freshly dead animals. Two vials of blood (in total 10–14 ml) should be collected from the jugular or caudal vein with a needle and vacutainer tube in mammals; and from the wing veins in poultry. Vials should be frozen in LN2 vapour phase and stored in LN2 (FAO 2012).

Tissue collection for DNA/RNA extractions or pathological examinations. Organ collection should take place at slaughtering houses and should be carried out by a necropsy technician or veterinarian in a predetermined order (**Leibniz Institute for Farm Animal Biol-**

ogy 2016). Pictures should be taken during the entire procedure. Organs should be rinsed with sterile PBS, and 1–2 cm^2 slices should be sampled and placed in 1 oz. Whirl-Pak bags, or 50 ml tubes to be immediately flash-frozen in LN2. Samples should then be stored on dry ice in coolers, which should be cleaned with 70% ethanol and transported in a well-ventilated vehicle to the laboratory. At the laboratory, they should be stored at -80 °C. Detailed procedures for different organs can be found in the **FAANG portal**: e.g., Tixier-Boichard and Fabre (2016), Burns (2018) and Jimenez and Plastow (2021).

Frozen samples (80–150 mg) should be crushed immediately for RNA extraction. DNA can be extracted using commercial kits. DNA is ideally stored in aliquots of 50 µl, with a concentration of 200 µg/ml. It can be stored at 4 °C for up to two months, otherwise it should be stored at -20 °C (for medium-term use) or lower (-80 °C or in LN2).

Wildlife

Conservation efforts for wild animals have so far included *in situ* support (e.g., habitat conservation), breeding programs at zoos and aquaria and DNA banking. The cryopreservation of blood/haemolymph, nucleotides, tissue, living cell lines, gametes and embryos has played a crucial role, among others, in assisted reproduction, animal and human medicine, evolutionary biology, systematics and conservation (Ryder et al. 2000; Bartels and Kotze 2006), as well as in biotechnology development. Animal biobanks can help to identify and prioritise which species are at risk of extinction and develop the most effective techniques for collecting, preserving, and storing genetic material (Breithoff and Harrison 2018).

Samples can be obtained during field trips or from zoological parks / breeding programs. There is no methodological difference when collecting samples from wild or captive animals. Nevertheless, the same field collection hygienic rules for livestock should be followed when sampling captive animals at zoos and aquaria (see livestock sample collection section above). Photographs of live animals, carcasses, and necropsies should also be taken from multiple angles before sampling.

The selected tissue preservation methods will depend on logistical factors, such as the duration of the field trip, convenient facilities, and taxonomic focus (Prendini et al. 2002). Wong et al. (2012) suggested a tissue sampling standardisation, ranging from one to four stars, to obtain the highest DNA/RNA quality, depending on the goal of the collection (e.g., cell line establishment, transcriptomics, whole-genome sequencing) (Table 2).

Table 2. Tissue standards for vertebrates but that may be extended to invertebrates, plants, and fungi.

	Four-star	Three-star	Two-star	One-star
Material origin	Fresh/live specimens	Fresh/live specimens	Salvaged, voucher	Salvaged, voucher
Tissue from multiple organs	Yes	Yes	No	No
DNA quality	1 mg (ca. 1 cm^3)	1 mg	>700 µg	≤ 700 µg
Reference species	Yes	No	No	No
Storage	Stabilisation reagents, LN	≤ -80 °C	≥ -20 °C	Ethanol
Packaging	Falcon tubes/ cryovials	Falcon tubes/ cryovials	Plastic bags	Plastic bags
Quality assessment	Barcoding	Not necessary	Not necessary	Not necessary
Target	Cell culture, DNA, RNA	RNA, DNA	DNA	DNA

Modified from Wong et al. (2012).

Ideally, tissues should be flash-frozen into dry shippers containing vapour-phase LN2 immediately after collection. However, LN2 tanks have to be taken to the field, if no sources are available at regular intervals, increasing costs and causing logistical obstacles that could compromise the expedition (Bennett 1999; Prendini et al. 2002; Gemeinholzer et al. 2010). This preservation method may be used when sampling captive animals or for short-term field trips. Tissues can be cut into small pieces and stored flash-frozen without preservatives, though adding preservatives prior to freezing may produce better long-term results (Mulcahy et al. 2016). They should be placed into a -80 °C freezer or LN2 tank as soon as they are removed from the dry shipper.

RNAlater, DNAgard Tissue, or AllProtect are non-hazardous stabilisation reagents that can be used as preservation solutions for several months at room temperature, but they are expensive (Zimkus et al. 2018). The most common preservation method is 95–96% ethanol, which can preserve DNA for long periods at room temperature and above (Zimkus 2018), although such temperatures will make high molecular weight (HMW) DNA applications very difficult. The use of denatured ethanol containing e.g., traces of benzene, or of lower concentrations (containing excessive amounts of water) or higher concentrations (potentially retaining chemical drying agents) of ethanol can degrade DNA (Neumann 2010). The use of DMSO/EDTA salt-saturated (DESS) solutions may be better for preserving HMW DNA in vertebrate tissues, whereas alcohol may be preferred for invertebrate tissues, although further research is needed (Mulcahy et al. 2016; Osting et al. 2020). Terrestrial invertebrates can also be trapped/preserved in food-grade propylene glycol or in ethylene glycol, although the latter poses a health hazard (Höfer et al. 2015; Liu et al. 2020). Vials should be totally filled to avoid specimen damage during transport. A minimum of a 3:1 volume ratio of ethanol to tissue is used for storage and efficient preservation. Note that to prevent watering down (due to the sample's body fluids), ethanol should be exchanged at least once shortly after sampling, e.g., on the next morning, otherwise (or on top of the exchange) a 10:1 ratio is recommended. When using alcohol, vials should be inspected the following few days after preservation and discoloured (typically yellow) alcohol should be discarded and replaced with fresh alcohol. Tissues should be cut into small pieces to allow rapid penetration of the preservatives (Prendini et al. 2002).

Transparent plastic vials with screw-caps are used to avoid ethanol evaporation. Stripes of Parafilm can be used to seal the cap, which might become loose during transport. Be aware that some types of polymers corrode when in contact with certain insect killing agents (Krogmann and Holstein 2010). Cryotubes are sometimes used for short-term storage during fieldwork in hot climates. However, they may not be the best choice, as they are designed for contraction during cryo-processes and high temperatures may allow for high ethanol evaporation losses (Neumann 2010) in some builds.

Invertebrates

Zooplankton. Refer to the Protista section for collection techniques (and see Wiebe et al. 2017 for further details). Once planktonic specimens are identified under a microscope, seawater has to be drained from the samples using a sieve with a smaller mesh size than the one used for field collection. Specimens should be preserved in 95% alcohol with a volume ratio 4:1. Alcohol should be replaced after 24 h, and then as many times as needed until the alcohol is clear. Samples should be stored at -20 °C (Bucklin 2000). Preservation buffers such as DESS, disodium EDTA, and saturated NaCl can also be used. Note that EDTA seems to be the relevant preservation agent in DESS, and that saturated salt alone is of limited efficacy (Sharpe et al. 2020). If samples are to be flash-frozen, they should be placed in ice baths or otherwise cooled during specimen examination, identification, and removal. Selected individuals should be stored in cryovials containing filtered buffer solution

or filtered seawater and stored in the vapour phase of LN2 (Bucklin 2000).

Marine invertebrates. Animals are usually captured by hand or by removing them from seabeds with a knife. As morphology varies among taxa, different tissue types are collected depending on the taxon. Some animals, such as anemones, brachiopods, bivalves, and polychaetes have to be relaxed before tissue collection. For a list of relaxants, see Templado et al. (2010). The following tissue sample procedures are described in more detail in O'Mahoney et al. (2015) (Table 3).

All invertebrates should be placed immediately in vials containing 95–96% ethanol (Krogmann and Holstein 2010). If the sample is voluminous or contains impurities such as leaves or algae, the ethanol should be replaced after six hours and again after 24 h (Aghová et al. 2019). The use of DESS buffer or RNAlater for samples containing calcium carbonate structures (e.g., shells) is not recommended, as they may be dissolved during storage (Knebelsberger and Stöger 2012).

RECOMMENDATION
Some invertebrates will either shrink/contract or harden and embrittle when placed immediately in 96% alcohol (Krogmann and Holstein 2010). Therefore, animals meant for morphological and molecular analysis should, as a compromise between methods, be first fixed in 80% ethanol for species determination and then in 96% ethanol for DNA fixation. Arthropods can usually be softened afterwards, e.g., through use of proteinase in non-invasive DNA extraction.

Table 3. Tissue sampling collection for different marine invertebrate taxa.

Taxon	Tissue sample
Sponges	Inner part of the body (ca. 5 mm^3)
Cnidarians	
- Anemones	- complete tentacle or a clip. Mind nematocysts
- Corals	- single polyps or a piece of branch containing a cluster
- Jellyfish	- oral arm or bell margin clip. Beware of poisonous species
Ctenophores	Comb-row tissues
Mollusks	
- Bivalves	- inner left and/or right mantle
- Gastropods	- muscular foot tissue. Avoid skin tissue, which is rich in PCR inhibiting mucus (mucopolysaccharides)
- Cephalopods	- arm tips (not the tentacle tips, which are longer), mantle. Minimise coloured skin tissue, which might inhibit PCR
Brachiopods	Muscular tissues holding the shell halves
Polychaetes	Middle body segments. The anterior and posterior ends are used for taxonomic purposes
Crustaceans	Middle leg (complete or terminal segment with no exoskeleton) or abdominal tissue, avoiding the gut
- Barnacles	- muscular peduncle or soft inner tissues
Echinoderms	Gonads
- Sea stars	- tube feet and arms
- Sea urchins and sand dollars	- muscular tissue surrounding Aristotle's lantern
- Sea cucumbers	- the gut, or inner body wall muscle tissues

For collection and preservation protocols of microinvertebrates (e.g., tardigrades, rotifers), refer to (Valdecasas et al. 2010), and for freshwater invertebrates, to Thorp and Rogers (2015).

Soil invertebrates. Animals may be collected individually with tweezers, by taking a soil sample, or by using (bait) traps. Bulk samples obtained by the latter two methods can be taken to the laboratory, where animals can be picked by hand, or by using the Hadorn method, a slightly heated metal dish, for inactive animals. Collected animals should immediately be placed in 95–96% ethanol (Aghová et al. 2019).

Insects and other arthropods. Arthropods are mostly collected by hand (e.g., individually with tweezers or an aspirator, or by netting) or by using different types of traps. The method of choice will depend on the biology and habitat of the species (Krogmann and Holstein 2010). For detailed collecting trap usage, see Achterberg et al. (2010), Grootaert et al. (2010), Floren (2010) and Steiner and Häuser (2010). For further collecting methods, refer to Whitman et al. (2019) and Santos and Fernandes (2021). Insects should also be placed in 95–96% ethanol, which works both as a killing and fixation agent. Do not use ethyl acetate as a killing agent as it quickly degrades DNA. If specimens are to be frozen at -80 °C, propylene glycol can be used as a cryoprotectant (Whitman et al. 2019). Place a single specimen into a vial, unless specimens are too small. Specimens should be kept in dark and cool conditions, and sunlight should be avoided (Krogmann and Holstein 2010). Whatman FTA cards can be used to sample haemolymph (Frozen Ark Project 2021a). Protocols for tissue selection for DNA analyses are provided by Pereira et al. (2022).

Millipedes possess repellent glands containing different substances (e.g., alkaloids, quinones or phenols) that are released into the ethanol and can eventually damage the DNA. Therefore, alcohol should be replaced within 4 h after first fixation. For larger invertebrates, such as spiders, the third right limb should be removed and placed in 96% ethanol for molecular analyses, and the rest of the body in 80-96% ethanol for morphological characterisation (Aghová et al. 2019).

Insect specimens may also be kept alive during transport and then fresh frozen in the laboratory (Krogmann and Holstein 2010). Insect freezing temperatures depend on the species, stage, and physiology, but they usually range between -4 °C and -22 °C (Fields et al. 2012). Usually, small species should be left frozen for at least 30 min, and large species for 24–48 h or even longer (Aghová et al. 2019; Whitman et al. 2019).

Vertebrates

In case specimen vouchers are needed, fieldworkers have to get appropriate training for euthanising animals, as it must be quick and as painless as possible for the animal (Hoffmann et al. 2010). Before going to the field, a literature review should be conducted for anaesthetics and analgesics used in the species of interest (Sikes and Gannon 2011). Cooperating with hunters, utilising fur harvest, and searching in town markets are other alternatives to obtain samples for genetic analyses. Non-invasive sampling should also be considered, especially when working with endangered species (see Lefort et al. 2022 for a detailed review).

RECOMMENDATION
Forceps for tissue manipulation should have smooth tips rather than serrated ones, to allow better cleaning of the tips and to avoid contamination from residual mucus/tissue adhering to the serration (Neumann 2010). Ideally, forceps are wiped clean with 96% ethanol and flame sterilised after each sample. Alternatively, hydrogen peroxide or 10% bleach solution can be used to sterilise instruments.

Sampling can also be opportunistic when animals are found dead, but the tissue quality will depend on the stage of decomposition (Aghová et al. 2019). Unexposed muscle tissue is usually sampled, but if the animal is already

rotting, an intact blood-coloured part should be detected and collected, or a dry gauze dressing pad should be used to wipe moist remains. Samples should be placed in double Ziploc bags and kept frozen if possible (Australian Museum 2019).

Collecting fresh blood is a common procedure applied to all vertebrates. Blood volume should not be more than 1–1.5% of the animal´s body mass (Gemeinholzer et al. 2010; Sikes et al. 2011). Blood can be collected in regular or in EDTA vials (heparin should be avoided, as this will inhibit PCR reactions (Woog et al. 2010)), on filter paper, or on FTA cards. Blood in vials should remain un-clotted and can be split into several aliquots to maximise the sample´s utility. FTA cards and filter paper only require one or very few drops of blood (up to ca. 125 µl), which should be allotted slowly and evenly across the paper and left to thoroughly dry for several hours or overnight. Cards should be placed in Ziploc bags and kept at room temperature. Avoid touching the inside pouch area of the cards with your hands, as well as rubbing or smearing the blood onto the card. If dried blood is found, wet isopropanol swabs should be used to wipe the blood smear. Swabs should be put in Ziploc bags (Australian Museum 2019; Frozen Ark Project 2021b, c). FTA cards can also be used for tissue and saliva sampling (Smith and Burgoyne 2004).

Vertebrate faeces can also be sampled for population genetic studies or diet studies. Various faecal sample collection protocols exist that include the use of ethanol, Queen´s College lysis buffer, DMSO, RNAlater, silica or drierite desiccants, or dry sampling on wax paper (Biswas et al. 2019). Samples should be kept in the dark until reaching the laboratory, where they should be stored at -20 °C until further processing (Biswas et al. 2019). More information can be found in Paupério et al. (2018).

If cell culturing is the main objective, tissue samples should be placed in conical plastic tubes (50 ml) containing a tissue-specific medium, foetal bovine serum, and antibiotics, instead of being placed in alcohol. Samples should be cooled (not frozen) and transported to the laboratory as soon as possible (within a few days). See the "culture preservation and storage methods" chapter for details.

Fish. Methods for sampling fish include nets, angling, spears, electrofishing, and fish traps. If animals are not euthanised, fin clips should be collected within 2 min. Afterwards, animals should be placed in a bucket until fully recovered to prevent injuries or predation while still sedated (Neumann 2010).

RECOMMENDATION
Note that electrofishing licences, spear gun and gill net permits, skipper licences, and diving certifications are additional documents that may be required to sample fish.

Muscle tissue without skin (ca. 5 mm^2) should be collected from above the right pectoral fin (behind and above the gill arch) or from the caudal peduncle, leaving the left side intact for imaging. The sampling area should be descaled and wiped clean with ethanol before taking the sample (Smith and Bentley 2018). The posterior area of pelvic fins, on the ventral surface of Chondrichthyes, is preferred for sampling (FAO 2016). The right eye and gill filaments can also be removed, as well as the caudal end of small larvae and juveniles (FDA 2011; Neumann 2010). For live Chondrichthyes, a dermal punch should be collected from the trunk area below the dorsal fin (FAO 2016). Specimens should be kept cold if tissue collection is delayed (Smith and Bentley 2018). Tissues should be placed in gasketed vials filled with 96% ethanol and stored in a cool place (Neumann 2010).

Viable tissue for cell culturing can be obtained from fin clippings. If possible, the tissue should be treated with polyvinylpyrrolidone (PVP) to reduce microbial contamination before any cryopreservation procedure takes place. Otherwise, decontamination should be carried out post-thaw and prior to establishing cell line cultures (Rawson et al. 2011).

Non-destructive sampling includes fish swabs, fin/tail clips, and scale collection. Swabs should be wiped only in the fish's mouth without touching any other surfaces. Swabs can also be wiped down from the pectoral fins to the start of the caudal fin if the fish´s mouth is too small for the swab. Clips can be taken using scissors to cut a piece of the pectoral or caudal fin (FAO 2016). Store samples in portable coolers. Scales can be loosened using the back of a scalpel below the lateral line and below the middle of the spiny dorsal fin. At least 15 scales should be collected for genetic analyses. Scales can be air-dried and stored in paper envelopes (FAO 2016) or kept frozen until transport (Australian Museum 2019).

Reptiles and amphibians. Sweep sampling, dip-netting, kick sampling, stovepipe sampling, and funnel trapping are among the different techniques to collect herpetofauna. Handling of captured animals is done by hand, hooks, tongs, and nooses to increase the safety of both the sampler and the specimen (Eekhout 2010). Field techniques are summarised in Bennett (1999) and Beebee (2010), and larval sampling in Skelly and Richardson (2010). Keep live animals in small containers with ventilation or in dry cloth bags (wet cloth bags can lead to suffocation), avoiding direct sunlight to prevent overheating if sampling does not take place directly after collection.

RECOMMENDATION

Be aware that only experienced and trained professionals should capture and handle venomous snakes. Moreover, amphibians should be handled carefully, due to the toxicity in their skins. Latex gloves are recommended, but if not available, hands should be washed thoroughly after manipulation, making sure to avoid contact with the eyes or mouth (Eekhout 2010). In addition, strict hygiene procedures should be followed when handling amphibians in the field, as a researcher can involuntarily become a vector for chytrid fungi among populations.

Muscle and liver are the most common tissues used in amphibians for genomic studies, whereas tissues from foot, tongue, skin, and gonads are used for cell lines (Zimkus et al. 2018). Blood samples are usually taken from the femoral or jugular vein, a carotid artery, the retroorbital space and the paired cervical sinuses in turtles; from the orbital sinuses in medium- and large-sized lizards; and from the internal jugular or caudal veins in crocodilians. Only large amphibians can bear blood sampling from the midline abdominal vein (Eekhout 2010), although new techniques using fibre-optic lights and doppler ultrasound may enable the method on small amphibians (Zimkus et al. 2018). Tail and toe clips are collected to obtain tissue, along with tail scute clips in crocodilians, scute scrapings in turtles, and tail crest nicks in lizards (Bennett 1999; Keller et al. 2014, Kucklick et al. 2010). Tadpoles and juveniles should be collected whole, as they yield low amounts of tissue (Zimkus et al. 2018).

All collected material, preferably tadpoles, kidneys, and eye tissue, intended for cell culturing should be stored just above 0 °C for no more than two days (Rawson et al. 2011). Subsequent steps can be found in the "culture preservation and storage" chapter.

Non-invasive methods include the sampling of reptile skin sheds, ventral scales from snakes, shell or scale remnants, cloacal and buccal swabs, and addled eggs (Bennett 1999; Eekhout 2010; Keller et al. 2014). Detailed necropsy, tissue and egg collection protocols focused on turtles can be found in Keller et al. (2014).

Birds. Mist nets are the most common method to capture birds. Collection techniques can be found in Bibby et al. (1998) and Woog et al. (2010). Birds can be released immediately after sampling.

For genetic studies in birds, blood is usually sampled. Blood can be collected from the right side of the bird's neck (jugular vein), the wing vein (brachial/ulnar vein), or the leg vein (medial metatarsal vein), depending on species and age (Gemeinholzer et al. 2010). Buccal swabs can also be taken following Vilstrup et

al. (2018). Swab samples can be stored at room temperature until extraction.

Feathers from the breast or back should be plucked, never cut, in the direction of growth, keeping the root attached. They should be placed in envelopes or Ziploc bags containing silica gel and kept at room temperature. The last 3 mm of the quill tip, where blood and skin cells are found, should be stored at -20 °C upon arrival to the laboratory. Addled or unhatched eggs should be carefully opened to collect their contents, which may be useful for monitoring pollutant concentrations (Walker et al. 2014). Transport eggs in foam padding coolers.

Mammals. Several methods can be implemented to collect mammals, which depend on the animal´s biology and its body size. For instance, small mammals (< 500 g) can be collected using Longworth or Sherman live traps, while Tomahawk and Havahart traps are used on medium-sized mammals, mist nets and Harp traps on bats, and purse seines and mechanical clamps with lines on marine mammals. Large mammals should be captured using remotely injected anaesthesia or analgesics. For detailed information regarding trapping methods, consult Kunz et al. (2009), Hoffmann et al. (2010), and Sikes and Gannon (2011). Trapped animals are usually alive, and small mammals may be euthanised only if taxonomic identification is problematic or if needed as reference material in collections. Otherwise, animals should be released at the capture site, after sampling and marking are complete. For euthanasia procedures, ethics, and treatment of animals in the field see Gales et al. (2009) and Sikes and Gannon (2011).

RECOMMENDATION
Use gloves and full protective gear, if possible, when handling animals either dead or alive, as they represent a health risk due to zoonotic disease transmission.

Blood, biopsy punching, and toe/tail clipping (exclusively for small mammals) are the most common methods to obtain DNA samples. Blood can be drawn from specific areas of the body, depending on the type of mammal (e.g., the ear vein in elephants, the femoral vein in primates or the antebrachial vein for bats). The punching method comprises punching holes or making nicks in the ear/wing of the animal (ca. 3 mm diameter for small animals; ca. 1–3 cm^2 for large mammals under anaesthesia). Toe clipping consists of removing the distal phalange bone of one limb, whereas tail clipping consists of cutting off a little portion of the distal tail (1–2 mm) with sharp scissors. Samples should be placed in vials containing 96% ethanol and kept cool (ca. 10 °C), not frozen (Gemeinholzer et al. 2010; Hoffmann et al. 2010; Kucklick et al. 2010). Further details on collection and storage protocols can be found in DBCA (2017).

Hunted and stranded animals or incidental takes can either be sampled in the field or at a laboratory facility for necropsy procedures. The following also applies to captive dead animals. Skin tissue (1 cm in diameter) should be sampled after hair removal and after the skin is wiped with alcohol. Blubber from marine mammals should not be sampled (Irish Whale and Dolphin Group 2008). Tissues from different organs (150–200 mg or 3 cm^3) can be collected and placed in 96% ethanol. If the ethanol looks cloudy, it should be replaced several times until clear. If live cell preservation is the main goal, skin samples should immediately be placed in freezing media and frozen using a Mr. Frosty or CoolCell container overnight at -80 °C (see culture preservation chapter) and stored at this temperature or in LN2 vapour-phase (Frozen Ark Project 2021c). Necropsy protocols for pinnipeds, sea otters and cetaceans can be found in Becker et al. (1991).

Non-invasive samples such as hair should be taken by pulling, never cutting, in the opposite direction of growth to collect the root. Keep hair in envelopes dry at room temperature with silica gel. Horn/tusk scrapings can also be kept at room temperature in sterile containers. In addition, saliva can also be collected using a swab to wipe the mouth mucosa (Australian Museum 2019).

Gamete banking

Apart from somatic cells, biobanks can also store gametes, gonads, and embryos, which can be used in assisted reproductive techniques (ART) to benefit population management strategies (Ryder et al. 2000). Be aware that ART protocols developed for one species cannot be applied to other species because reproductive mechanisms vary among species (Silva et al. 2017). Understanding the reproduction seasonality and reproductive physiology of the species of interest is therefore crucial to increase success in collecting and preservation (Charlton et al. 2018).

Moreover, gamete cryopreservation protocols in wild species remain difficult, not only because this type of material is scarce, but also because protocols also tend to be species-specific. Oocyte freezing or embryo-based technologies are currently not used in wildlife management, due to the lack of knowledge about the species' biology, and due to the lack of expertise and facilities (Comizzoli 2017). Recovering gametes from living animals is costly, as it requires specialised staff and equipment, and hormonal stimulation methods, as well as being risky because anaesthesia and surgery sometimes are needed. Gametes are therefore usually obtained from castrated or euthanised animals (Fernandez-Gonzalez et al. 2019).

RECOMMENDATION
Methods for species identification should be available at the cryopreservation facilities to reconfirm species name.

Invertebrates

Marine invertebrates can be collected by hand in the field in the intertidal zone or by using scuba diving (Lessios et al. 2013; Adams et al. 2019), or can be obtained from mariculture farms (i.e., commercial species). Ideally, gravid organisms should be collected, but it may be challenging to detect them. Although maturing organisms in the laboratory is possible, it is often a time consuming and costly process. Hence, it may be necessary to sample several times during the breeding season (Adams et al. 2019), due to slight fluctuations in temperature or food availability, among other factors. Specimens have to be transported alive at a constant temperature, and carefully carried, as stress (e.g., vibrations, temperature, desiccation) can make them release gametes. Individuals can be maintained together in husbandry under laboratory conditions at the highest standard (van der Horst et al. 2018; Adams et al. 2019; Zuchowicz et al. 2021) or separated by sex whenever possible (Paredes and Costas 2020). When housed together, spontaneous spawning can occur. If so, as soon as spawning is observed, the individual should be immediately put aside into an independent container, not only for gamete collection, but also to evade simultaneous spawning. Gametes can also be collected by dissecting the animals (i.e., ideal for concentrated non-activated sperm), or by inducing spawning either chemically (e.g., hormones or concentrated salt solutions) or via stress (e.g., temperature changes, vibration) (Williams and Bentley 2002; Dale 2018; van der Horst et al. 2018). Spawning and gamete collection protocols for mussels, sea urchins and sea cucumbers can be found in Paredes (2020). In crustaceans, it is possible to apply pressure on the body to obtain gametes or use electroejaculation (Aquino et al. 2022). Note that the choice of procedure will depend on the species, because the method will affect the viability of the cells. Pooling of sperm or eggs is possible for cryopreservation purposes.

Collection and maintenance methods for polychaetes can be found in Watson (1997), Williams and Bentley (2002) and Caldwell et al. (2011a); for echinoderms in Caldwell et al. (2011b), van der Horst et al. (2018) and Adams et al. (2019); for marine molluscs in Fitzpatrick et al. (2012), van der Horst et al. (2018) and Beirão et al. (2019); for crustaceans in Rendón-Rodríguez et al. (2007), Beirão et al. (2019) and Aquino et al.

(2022); for nemerteans in Stricker et al. (2012); for ascidians in Yoshida et al. (2003); and for corals in Zuchowicz et al. (2021). Research on other invertebrate phyla is still scarce or non-existent (Poccia 2013; Zuchowicz et al. 2021). So far, only culturing and gamete morphology observations have been accomplished for tardigrades (Sugiura et al. 2022 and references therein).

A special issue, focused on collection, handling, and storage of gametes and embryos from marine invertebrates, has been published in the **journal Animals**. Further information can be found in Lewis and Ford (2012).

Insects can be reared in colonies or in small stock groups where mixed adults are free to mate to obtain eggs and embryos (Vasudeva et al. 2019; Zhan, et al. 2021). Sperm from ants and bees can be collected from freshly killed mature males by squeezing the abdomen until semen appears on the endophallus (Pearcy et al. 2014; Hayashi and Satoh 2019). Guidelines for mosquito colony maintenance can be found in FAO/IAEA (2017). Spiders should be maintained in individual tubes for some days to ensure that palps are recharged. Pedipalps should be removed, and the genital bulb separated from it to obtain the sperm (Gabel and Uhl 2013).

Vertebrates

Sperm collection

RECOMMENDATION
Seminal volume, motility, and progressive motility should be assessed immediately after collection to secure that only good quality material is stored.

Fish. Males are usually euthanised to obtain sperm from testicular tissue, as collecting sperm from live fish remains challenging (ERFP 2003; Rawson et al. 2011). In some cases, sperm can be collected after using hormones and by abdominal massage (Tiersch 2001). *In vivo* and post-mortem sperm extraction from sharks and rays can be found in García-Salinas et al. (2021). Beirão et al. (2019) provided a review about sperm handling in fish.

Reptiles. In snakes, sampling should take place during mating season. Animals should be sedated using local anaesthesia before proceeding. The skin around the cloaca should be cleaned and semen collected directly from the genital papilla using a needleless syringe. Stimulation is achieved by massaging the ventral portion of the snake toward the cloaca. Anaesthesia helps to provide better control over the cloaca, avoiding contamination with faeces and urine (Zacariotti et al. 2007). For semen collection protocols in other reptiles, refer to López-Juri et al. (2018) and Martínez-Torres et al. (2019) for small- and medium-sized lizards, to Assumpção et al. (2017) for caimans, to Zimmerman et al. (2013) for iguanas, and to Zimmerman and Mitchell (2017) for tortoises.

Amphibians. Sperm can be collected by catheterisation, stimulation of urination or by gently massaging the abdominal area to help produce spermatophores, after hormone induction (Browne and Figiel 2011). The latter method requires that the animal is placed on a Petri dish to avoid losing the sperm through spillage. In salamanders, the spermatophore should be pipetted directly from the cloaca. Sperm can also be obtained by macerating the testes in amphibian ringer solution (ARS) (Browne et al. 2019; Della Togna et al. 2020). Samples should be stored at 4 °C for up to seven days (Zimkus et al. 2018). A list of sperm recovery from various anuran species can be found in Zimkus et al. (2018) and Figiel (2020). Further collection protocols and storage can be found in Browne et al. (2019) and Calatayud et al. (2022). To improve or find the optimal cryopreservation method, follow Della-Togna et al. (2020).

Birds. Sperm is collected by cloacal massaging (Girndt et al. 2017; Kucera and Heidinger 2018; Özkök 2022), but its processing still requires further research, as bird sperm is fragile (Comizzoli 2015). Refer to Gee et al. (2004) and Samour (2004) for further general details on semen collection. Semen collection methods for parrots can be found in Lierz et al. (2013) and for ostriches in Malecki et al. (2007).

Mammals. One of the most common methods to collect sperm is electroejaculation, which has been developed for domestic species and has been successfully used on wildlife species, from marmots to tigers. Appropriate rectal probes are necessary, and it may be done under anaesthesia (Laura Graham, pers. comm). However, it is an expensive method, and the collected semen may be contaminated with urine, because of bladder contractions (Charlton et al. 2018). Urethral catheterisation under anaesthesia is an alternative which does not require specialised equipment or electrical stimulation but poses a greater risk of injury to the urethra, potentially impairing future breeding. So far, this method has not been successful in small mammals (Laura Graham, pers. comm.). Triggered or untriggered spontaneous ejaculation, rectal massages, and manual stimulation of the penis, are other methods to collect sperm from trained animals. Sperm can also be recovered post-mortem from the epididymis or testicular tissue (Roth et al. 2016; Charlton et al. 2018). See Silva et al. (2016) for a detailed description of sperm collection.

A review of germplasm collection and preservation methods used on different mammal species can be found in Durrant et al. (1990), Charlton et al. (2018), and Beirão et al. (2019). For species-specific sperm collection protocols for South American wild species, refer to Silva et al. (2016), for marmoset monkeys, to Kuederling et al. (1996), and for other primates, to Schaffer et al. (1989). Protocols for Tasmanian devils can be found in Keeley et al. (2012), for wolves and dogs in Christensen et al. (2011), and for belugas in O´Brien et al. (2008).

Oocyte collection

Ideally, ART protocols should be in place before oocyte harvesting and preservation (Saragusty and Arav 2011). The collection of oocytes from wildlife under anaesthesia can be performed laparoscopically by trained staff using hormonal stimulation of the ovaries. This procedure is still in the research phase for many wildlife species. Post-mortem collection of oocytes is possible, although determining the ideal conditions for temporary storage appears to be species specific and is still in the research phase for most species (Laura Graham, pers. comm.).

In amphibians, eggs can be collected during oviposition or by manual stripping, which involves pushing on the abdomen towards the cloaca (Zimkus et al. 2018). Developing hormonal treatments for egg collection is challenging, as females need administration of priming doses, due to their complex hormonal cycles (Della-Togna et al. 2020). Oocytes can also be collected post-mortem and kept at 4 °C for up to five days.

In felids, ovaries can be removed from euthanised animals and kept cool in a physiological saline solution such as PBS (Jewgenow et al. 2017; Fernandez-Gonzalez et al. 2019). Cortical ovarian tissue should be cut into slices and cryopreserved. However, post-thaw oocyte survival included in this tissue has not yet been assessed (Jewgenow and Zahmel 2020). The use of ovarian preantral follicles in mammals can be a successful alternative to obtain mature oocytes because they comprise most of the ovarian cortex tissue (Campos et al. 2019). This procedure is challenging, as follicles may result in suboptimal oocytes or show resistance to *in vitro* methods (Saragusty and Arav 2011). Culture conditions for oocyte growing are mostly unknown but some advances have already been achieved for several species of felids, primates, and dasyurid marsupials (Campos et al. 2019).

Embryo Collection

Embryo collection and cryopreservation have been reported in domestic animals which are used as models to design techniques for wild species. Two problems remain: first, the same protocol may not work for another species, even if closely related; and second, naturally produced embryos in wildlife should continue their development rather than being collected for storage (Saragusty and Arav 2011).

Embryonic stem cells. So far, true embryonic stem cells have been identified in laboratory species, such as mice and primates, and can be

obtained from the blastocyst's inner cell mass or early-stage germ cells. These cells can be frozen, thawed, and grown through numerous cell cycles (FAO 2012). For instance, PGCs, which are the embryonic precursors of gametes, may be ideal for fish surrogate production, offering a solution for the management of species with reproductive failures, or for those species with long maturation periods (Robles et al. 2017).

Parasites

Animal parasites

Parasite collections are underrepresented in biodiversity biobanks, in contrast with other taxa such as mammals or insects (Bell et al. 2018). The most effective method to collect parasites is as soon as the host animals are euthanised and processed in the field (Gardner 1996), otherwise they should be kept frozen until further analyses (Galbreath et al. 2019). Make sure to follow animal welfare regulations and approved euthanasia/necropsy protocols depending on taxon. Parasites can also be collected from fur harvest carcasses, game, and road kills, as well as from fish markets and fresh faeces (Julca et al. 2014, Kutz et al. 2014; Caira et al. 2017; Schwartz et al. 2020).

It is also possible to obtain certain parasites from live hosts subdued by light-anaesthetising or by holding (Gardner and Jiménez-Ruíz 2009). Note that mainly ectoparasites will be collected by this method.

Faeces from small mammals should be taken from the rectum and placed in a Wheaton snap-cap vial half-filled with 2% potassium dichromate $K_2Cr_2O_7$ to obtain coccidian parasites, helminth eggs or protozoan oocysts (Gardner and Jiménez-Ruíz 2009). Fresh faecal pellets from large mammals (e.g., ungulates) should be stored in plastic bags with silica gel. Faecal material can also be preserved in 96% ethanol or frozen until processed (Galbreath et al. 2019). Nematodes can be collected from faeces using the Baermann beaker method (Forrester and Lankester 1997; **Western College of Veterinary Medicine**, University of Saskatchewan) or by following a modified version by Galbreath et al. (2019).

Blood-borne parasites can be examined either by preparing blood smears in the field or by collecting blood with FTA paper, Nobuto filter strips, or hematocrit tubes, which can be sealed with Critoseal (Gardner and Jiménez-Ruíz 2009; Galbreath et al. 2019).

Ectoparasites

Arthropods, such as mites, fleas, ticks, and lice, as well as botflies and warble larvae are the most frequent and abundant ectoparasites. Each captured host, either dead or alive, should be placed in a thin plastic bag, to avoid cross-contamination and losing the ectoparasites (Gardner 1996). Bags are placed in glass jars containing cotton moistened with chloroform to kill the ectoparasites. Ears, eye areas, skin folds, pelvic cavities, gills, scales, and feathers should be examined using a dissecting microscope, ideally over a sheet of white paper. For mammals, the pelage should be checked by combing the hairs of the dorsal and lateral body surfaces backward. Ectoparasites found in the skin should be pried to keep their mouthparts, otherwise they can be removed with a swab, forceps, or a needle dipped in ethanol, and stored in individual vials containing 70% or 96% ethanol (Gardner 1996; Gardner et al. 2012; Galbreath et al. 2019). Bags with ectoparasites should be washed with 70% ethanol (2–6 ml), and content drained and transferred into a Whirl-Pak, followed by final storage in a vial (Gardner 1996).

RECOMMENDATION
Standard cloth bags may retain ectoparasites from previous uses for extended periods of time and should therefore not be used during fieldwork. Otherwise host transfer must be assumed to occur (Gardner et al. 2012). Clean killing jars properly after every use to avoid the presence of previous ectoparasites.

Vertebrates can be parasitised by muspicioid nematodes that are found within skin nodules. Affected skin areas should be photographed beforehand. The nodules should be disinfected and then opened with a sterile dissecting pin to remove any nematodes and transfer them to a saline solution (Gardner and Jiménez-Ruíz 2009). If the animal host is anaesthetised, nodules can be surgically removed and placed in 70% ethanol. Skin should be closed using tissue glue (e.g., Vetbond, 3M) (Holz et al. 2018).

RECOMMENDATION
Cross-referencing will be more accurate when the host species name, the host identification number, and the body part where the parasites were found are stored together with the collected parasites in bags or vials.

Endoparasites

After the ectoparasite sweep is completed, the host necropsy protocol may follow. It is crucial that the specimens are processed immediately, as proteolytic enzymes will begin breaking down helminths and hooks on the scolex of cestodes can fall off within minutes after the host dies (Gardner and Jiménez-Ruíz 2009; Galbreath et al. 2019).

Organs of small animals (< 10 kg) should be removed and placed in a Petri dish, containing water or physiological saline. Every single organ should be separately examined for parasites using a dissecting microscope, and they should be preserved and stored in distinct vials. Do not mix parasites from different organs (Gardner and Jiménez-Ruíz 2009). Galbreath et al. (2019) suggested a protocol for parasite sampling from mammals which can be included during mammal surveys, and it can be applied to any other vertebrate.

Parasite preservation should be done meticulously, to avoid morphological damage and DNA deterioration. Tapeworms (cestodes), flukes (trematodes), and thorny-headed worms (acanthocephalans) should be relaxed by placing them in distilled water or any other freshwater. Water causes an osmotic shock leading to death of the worm. Nematodes, however, should be placed in saline water, otherwise they will burst. Hot water and heat-killing methods may also be performed for helminth preservation (Cook et al. 2016; Galbreath et al. 2019). Exposure to formalin or acids should be avoided (Gardner and Jiménez-Ruíz 2009). Specimens should be stored in vials containing 80–96% ethanol as a starting concentration, as distilled or saline water will dilute the ethanol to ~70% (Lutz et al. 2017). Samples should be stored at -85 °C in an ultra-low freezer (Gardner et al. 2013) or at -20 °C for short-term storage (Yuan et al. 2016).

RECOMMENDATION
After each animal necropsy, rinse dissecting instruments in 70% ethanol made with a solution of 10% bleach and dilute high-phosphate detergent, then rinse in distilled water, and wipe dry with a clean tissue to avoid contamination with blood or other sources of foreign DNA. Do not forget to use surgical gloves and laboratory coats during necropsy procedures!

Digenean trematodes comprise the most common eukaryotic pathogens in aquatic ecosystems (Selbach et al. 2020), causing waterfowl die-offs (Roy and St-Louis 2017) and severe diseases in humans. Usually, the final host is a large vertebrate (e.g., humans) and adult parasites are therefore inaccessible. Instead, larval stages (miracidia and cercariae) have to

be collected from intermediate hosts such as snails or crustaceans (Emery et al. 2012) For instance, freshwater snails are regularly monitored in Africa to be examined for flukes such as *Schistosoma*.

RECOMMENDATION
When travelling long distances, snail jars should be wrapped with damp cloths and placed in a cool box for transport. This will keep the snails cooler; decreasing death and stress of the snails.

Snails are usually collected by hand or using metal mesh paddle scoops. Snail species should be identified and then placed in plastic jars filled with water for later processing. Once in the laboratory, they are rinsed in de-chlorinated water to remove any surface- adhering organisms. Snails should be placed separately in cups with filtered freshwater and exposed indirectly to a light source for two to five days to induce cercarial emergence/shedding at 20 °C. Cercariae should be preserved either in 96% ethanol (Selbach et al. 2020; Duan et al. 2021) or on FTA cards using a Gilson P20 pipette set at 3–4 µl (Emery 2012). FTA cards should include 20–60 cercariae and they should not touch each other. The FTA card should be dried for one hour and placed in a plastic sealable bag. The DNA should be stable at ambient temperature. Snails are preserved in 96% ethanol (Emery 2012).

Invertebrates are also affected by gregarine parasites. After field collection, host animals can be maintained in laboratory colonies for further analyses. Insects can be placed into individual jars to collect faeces that might contain gametocysts. Protocols for collecting and culturing gametocysts can be found at Hotel Intestine (http://science.peru.edu/gregarina/Technique.html). At the laboratory, insects, annelids, crustaceans, echinoderms (sea cucumbers), and mollusks can be euthanised and dissected to examine for endoparasites using a sterile elongated Pasteur pipette, under a stereomicroscope. The gastrointestinal tract should be removed and placed in a saline mixture (1 volume NaCl: 100 distilled water), PBS, or filtered seawater, depending on the species. Gregarines should be washed with PBS, autoclaved filtered seawater, or 90% ethanol at least three times to remove host tissue and bacteria. Specimens should be stored in 90% ethanol at -20 °C prior to DNA extraction (Clopton 2009; Wakeman and Leader 2012; Creigh et al. 2018; Florent et al. 2021).

Museum specimens field-fixed in 10% formalin are another good source to survey for parasites. Internal organs should be removed and dissected to detect and identify helminths by stereoscope microscopy (Gómez et al. 2020).

When both DNA-based and morphological analyses are required, a small portion of the middle or posterior region of the helminth can be cut out and stored in 96% ethanol at -20 °C, while the scolex and the rest of the strobila can be fixed and kept as a hologenophore (Astrin et al. 2013). In this way, the genotype will match to its corresponding phenotype (Choudhury 2020).

Plant parasites

Crops and grasses can be parasitised by nematodes of the family Heteroderidae. When looking for nematodes, the upper soil layer (3–6 cm) should be removed before sampling (van Bezooijen 2006). Do not collect soil that is too dry, as dormant nematodes may already be damaged (Shivas et al. 2005). Soil core samples should be collected after harvest at a depth of 15–30 cm from several spots around the field in a zigzag pattern and taken from around the plant root. Entire plants that show symptoms of nematode infection can be also sampled using the "Stretcher" method (van Bezooijen 2006).

Samples should be placed in individual plastic bags or paper bags coated with paraffin to retain moisture. Samples should be kept cool and out of direct sunlight, which can cause nematodes to die from shock. Samples should be transferred to the laboratory to be processed as soon as possible (van Bezooijen 2006; Jagdale and Arnold-Smith 2011; Smiley et al. 2017). Several techniques are used for extracting nema-

todes from both plant material and soil using either a Whitehead tray or a Baermann funnel, or sieving, decanting, and flotation methods (Shivas et al. 2005). Refer to van Bezooijen (2006) and Shivas et al. (2005) for protocol details.

Preservation methods are identical to the ones mentioned for nematodes parasitising animals.

RECOMMENDATION
Do not forget to include information regarding location, soil type and texture, plant symptoms (e.g., yellowing, necrosis, root rotting, galling, wilting), and cropping history together with the sample.

Environmental samples and community DNA

DNA is shed from all organisms into the environment and deposited as eDNA, which can be collected from non-biological substrate, including soil, air, and water (Howell et al. 2021; Clare et al. 2022). eDNA analysis is a non-invasive technique, as it can detect organisms without the need to capture species for sampling (Johnson 2017). This approach has the potential to identify either specific species using qPCR and ddPCR or whole communities using amplicon metabarcoding or, more recently, shotgun sequencing implemented in high-throughput sequencing platforms (e.g., Pacific Biosciences, Oxford Nanopore) (Hansen et al. 2018; Harper et al. 2019; Bell et al. 2021). It is possible to detect and to find enough eDNA both from microorganisms and macrobiota, although eDNA from the latter tends to decay faster because it is no longer found within intact cells (Thomsen and Willerslev 2015; Ruppert et al. 2019; Howell et al. 2021; Holman et al. 2021). Therefore, it is recommended to follow guidelines used for ancient DNA analyses (Lynggaard et al. 2022).

No standard methodology for eDNA can be applicable because studied sites and target taxa are unique (Ruppert et al. 2019). However, several factors should be considered, regardless of the habitat type (water, soil, sediment, air): sampling at different locations/strata and seasons, determining the physical and chemical properties of the substrata, as well as understanding the species ecology, habitat suitability, life stage, activity, and behaviour, will be essential to minimise false negatives and increase detection probability of wanted species (Harper et al. 2019). Note that these factors may be challenging in highly diverse habitats, where a large number of species may be detected (Vera Zizka, pers. comm.).

As eDNA is a recently emerging technique, there is still room for improvement in capturing enough eDNA, preserving eDNA samples before extraction, and lowering contamination risks from collection to extraction of eDNA (Spens et al. 2017). Hence, certain recommendations should be followed when dealing with eDNA and metabarcoding (Carim et al. 2016; Thomsen et al. 2016; Furlan et al. 2020; Banerjee et al. 2021; Howell et al. 2021):

1. Avoid having contact with targeted species and substrate before sampling.
2. All sampling material including filters, filter holders, tubes, and bags, should be clean (DNA-free) and sterilised. Decontaminate tools again after each use.
3. Avoid cross-contamination both in the field and at the laboratory. Do not re-use equipment, gloves, or special clothing between sites.
4. Use appropriate negative controls (i.e., blank filters) for sample collection, handling, transport, filtration, and DNA extraction to identify potential contamination. Also use replicates (ca. 3 per site).
5. Define high laboratory standards and stick to them.
6. Be aware of false negatives—species considered to be absent when in fact present—

and false positives–species considered to be present when actually absent. Also, consider that environmental transfer may occur, due to DNA dispersal by wind or water from one site to another one.

7. Although not part of laboratory protocols, be aware of DNA misidentifications when obtaining metabarcoding data. This error may happen when a sequence is assigned to the wrong taxon (due to, e.g., errors in reference data bases or insufficient reference data), or during the sequencing process, where tag jumps can occur (Schnell et al. 2015). Axtner et al. (2019) and Bohmann et al. (2022) suggest some strategies to mitigate eDNA metabarcoding errors.
8. Using a multigene metabarcoding approach would be ideal to improve taxa/undefined systems representation and resolution since databases may not be completed and are not exempt from errors.
9. Collect and sequence tissue samples from national history museums if common species are known in the studied area but are not represented in the databases.
10. Resample locations if unreliable results are obtained.

Further recommendations to avoid contamination when collecting samples can be found in Harper et al. (2019: online resources). Some guidelines and protocols have already been developed (Goldberg et al. 2016; ten Hoopen et al. 2017; Başoğlu et al. 2017; Egeter 2018; Przeslawski et al. 2018, 2019; Taberlet et al. 2018; Pawlowski et al. 2020; Bruce et al. 2021; Xing et al. 2022) and should be considered when designing eDNA projects. Sampling methodology will vary depending on the features of the environment, as well as on the research priorities (Goldberg et al. 2016). Thomsen and Willerslev (2015) offer a very detailed review and a summary of studies performed in different source samples that can help as a guidance. Furthermore, the European Committee for Standardisation is working on the prEN 17805 standard, water sampling for capture of macrobial environmental DNA in aquatic environments (CEN 2022), so parts of the eDNA methodology for water samples may be standardised soon.

This section focuses only on specific ecosystems and does not include eDNA collected from microbiomes (e.g., faeces, saliva), as these are found in the animal section of this chapter. Permafrost samples and sedaDNA can be found in the palaeontological sampling section.

Aqueous samples

No general guidelines exist for sample volume, depths, and water quantities. However, 500 ml – 2 l is the usual standard sampling volume from streams, rivers, lagoons, and seawater (Shu et al. 2020). Common sampling containers are 1-l HDPE bottles (Nalgene) or 5-l buckets, which should be decontaminated by soaking them in 10% bleach (sodium hypochlorite, NaOCl) for at least 30 min to remove all traces of DNA. Containers are then rinsed with distilled water to remove residual bleach (Eichmiller et al. 2014; Spens et al. 2017).

Surface water samples are the most widely used sample in eDNA and are taken by partially submerging a sample bottle or a sterile 60 ml Luer lock syringe to collect water from the top few cm (~10 cm) to 6 m. Sub-surface samples (~50 m) can be taken using a stainless steel Van Dorn sampler (Wildlife Supply Company) (Eichmiller et al. 2014) or automated samplers (Formel et al. 2021), whereas a rosette sampler or 5 l- Niskin bottles attached to remotely operated vehicles are used for deeper sampling (>1,000 m) (Everett and Park 2018; Laroche et al. 2020; Canals et al. 2021).

RECOMMENDATION
Note that high nutrient inputs, high turbidity, high temperatures, low pH, and high solar radiation can negatively affect eDNA, causing faster degradation, and hence reducing the amount of eDNA available within a waterbody.

Water samples should ideally be processed on-site, but if this is not possible, they have to be placed on ice and remain refrigerated (4 °C) to avoid DNA degradation. They should be processed within 24 h of reaching the laboratory (Hinlo et al. 2017; Harper et al. 2019; Shu et al. 2020; Bruce et al. 2021), otherwise, preservation buffers should be added immediately after collection (Mitchell and Takacs-Vesbach 2008; Williams et al. 2016; Kumar et al. 2020). Small volumes (<100 ml) collected with syringes can be stored in sterile Whirl-Pak until processed (Torresdal et al. 2017). The addition of 0.01% benzalkonium chloride can also mitigate microbial growth in the water samples (Yamanaka et al. 2017; Kumar et al. 2020).

Samples from the same waterbody with no stratification can be pooled and processed (preservation, eDNA capture, analysis) as one single sample or they can be treated as independent replicates. The latter should be preferred to increase species detection or if the aim of the project is to establish species distribution and habitat use (Harper et al. 2019; Shu et al. 2020).

Filtration and ethanol precipitation are the most used methods for eDNA capture (Hinlo et al. 2017; Harper et al. 2019). The precipitation method uses ethanol (twice the sample volume) or isopropanol (same volume as the samples with sodium acetate to precipitate nucleic acids in the water sample, but it is only feasible for a small water volume (<100 ml)) (Kumar et al. 2019). Samples can be kept at room temperature for up to seven days (Harper et al. 2019; Wang et al. 2021). Otherwise, they can be kept at -20 °C until DNA extraction (Egeter et al. 2018).

Filtration is a more effective method, as it can process larger volumes of water (0.5–2 l), and thus produce higher amounts of eDNA (Hinlo et al. 2017; Egeter et al. 2018). Filters are made of diverse materials, such as glass fibre, polycarbonate, and cellulose nitrate, and have diverse pore sizes, ranging from 0.22 µm to <10 µm, with 0.45 µm being the most used pore size (Egeter et al. 2018).

Two types of filters can be used: fully capsule-enclosed filters (e.g., Sterivex-GP) or open filters (Bruce et al. 2021). Filters enclosed in capsules reduce handling and contamination risk, as capture, storage and extraction take place within the capsule (Spens et al. 2017; Shu et al. 2020). Sterile syringes (e.g., 50 ml Luer-lock syringe) are used to slowly push water through the filter without tearing it (Holman et al. 2019). Any remaining water in the capsule should be removed with the syringe by pushing air through the filter. Preservation buffers (1.5–2 ml) can be added to the inlet end, and both the outlet and inlet ends should be closed with caps and sealed with parafilm. Capsules should be inverted vigorously and can be placed in -20 °C until extraction with or without preservation buffer (Spens et al. 2017; Holman et al. 2019). Another alternative to fully encapsulated filters is the use of self-preserving filters, which are compatible with suction pumps (e.g., Smith-root) and capture eDNA via desiccation at ambient temperature (Thomas et al. 2019).

RECOMMENDATION

Capsule-enclosed filters with a preservation buffer (RNA later, CTAB, ethanol 95–100%, Sarkosyl or Longmire's buffer) seem to be the optimal procedure. They can retain eDNA integrity and can be stored at room temperature for at least two weeks, making them ideal for remote and harsh fieldwork, as less equipment is required and are easy to transport (Spens et al. 2017; Mauvisseau et al. 2021).

Open filters require handling; thus, water samples should be filtered using sterile disposable filter funnels, and a vacuum or peristaltic pump (e.g., Nalgene 250 ml) (Spens et al. 2017). Filters should be removed with sterilised forceps and then folded in quarters with the filtrated side facing inwards (Carim et al. 2016). Filters can be placed dried or with preservation buffers in 5 ml - microcentrifuge tubes (e.g., DNA LoBind) or in Petri dishes, and frozen immediately at -20 °C (Spens et al. 2017). Alternatively, filters can be placed into clean

plastic bags or tubes containing silica beads, where they can remain for several weeks. However, samples should ideally be processed within the first two weeks after collection (Carim et al. 2016; Sales et al. 2019). Once collected, eDNA samples should be kept cool, dry, and in the dark (Spens et al. 2017).

RECOMMENDATION
Biodiversity detection can be affected by eDNA capture methods, as well as by material and filter pore size, which affect filter efficiency, and hence, DNA yield. These features should be considered when designing eDNA studies.

The use of sponges (Porifera) as natural samplers can also be a powerful and attractive method to detect eDNA from aquatic biodiversity. With further optimisation and validation, it will have the potential to filter more water and hence, to recover eDNA more efficiently than traditional methods (Mariani et al. 2019; Turon et al. 2020; Cai 2022; Harper et al. 2022).

Ponds and lakes. Lentic waters are basically motionless, allowing eDNA to accumulate over time (Harper et al. 2019), yet eDNA has a patchy distribution in such waterbodies (Eichmiller et al. 2014). Moreover, the presence of fallen trees and dense aquatic vegetation can be barriers to eDNA dispersion. Hence, samples should be collected at different spots, including underneath and around barriers, as well as at different depths to increase species detection (Harper et al. 2019). Filters can become blocked, when working in turbid waters, because of high levels of suspended solids and organic debris. Centrifugation, increased filter pore size, or pre-filtering will then be required (Harper et al. 2019; Bruce et al. 2021). An alternative is to use the precipitation method (Egeter et al. 2018).

Although sampling should be done at many different locations, limited accessibility (e.g., dense vegetation, or high steep banks) can hinder its optimisation. Sampling poles and boats can be used to enable water sample collection, but cross-contamination may occur between ponds. Several parameters should be recorded for better assurance and resolution of eDNA detection: 1) The total size of the pond perimeter, 2) the pond proportion that was accessible, 3) the water volume sampled and, 4) the number of samples and the distance at which these were taken (Harper et al. 2019).

It is important to note that terrestrial animals might transfer eDNA from one waterbody to another, causing false positive detections. (Harper et al. 2019).

Streams. eDNA is distributed more evenly in rivers due to the turbulent flow of streams, hence surface samples should be sufficient for eDNA detection (Shu et al. 2020). Consider sampling either downstream or upstream of tributaries, as well as across the river breadth. Note that eDNA may be diluted while moving downstream. Eddies or splashes should be avoided, as the water sample can get contaminated (Sales et al. 2019).

Carim et al. (2016), Lehmann (2016), and Pawlowski et al. (2020) developed sampling protocols for small creeks and rivers. The latter also includes step-by-step photographs of the whole sampling procedure. Kumar et al. (2022) provide protocols for both turbid and clear-water environments.

Oceans. eDNA concentration is usually low due to dilution (Bruce et al. 2021), plus eDNA can only be detected at the depth where it was released or deeper. Hence, vertical profiles play an important role in oceanic diversity detection (Canals et al. 2021). Furthermore, recovered eDNA will mainly represent local fauna, as eDNA does not exhibit a long-distance dispersal (Thomsen et al. 2016). Water samples obtained using Niskin bottles should be transferred to sterile 1 l bottles and stored at -20 °C until filtering is done at the laboratory (Thomsen et al. 2016).

It is important to note that current databases have a low representation of deep-sea organisms, hampering the analyses and further implementation of conservation measures (Laroche et al. 2020). However, attempts have been made to centralise genomic data from marine organisms (i.e., http://reefgenomics.org) (Liew et al. 2016).

RECOMMENDATION
All water samples have to be processed before sediment samples.

Throughfall in forest ecosystems and rainwater sampling can be revised in Šantl-Temkiv et al. (2018) and Ladin et al. (2021).

For protocols for other types of freshwater bodies (e.g., subterranean water, anchialine habitats, temporary, small water bodies) refer to Valdecasas et al. (2010).

Sediments

Sediment samples offer two advantages over other environmental sample types: first, eDNA can attach to particles in the sediment, providing a higher concentration that makes it easier to detect. Second, as eDNA can persist longer in this substrate, it is ideal to study past and current events, along with DNA transport and removal. However, this can also be a source of false positives, if past species are considered present in current times (Sales et al. 2019). A possible solution is to use radiocarbon dating techniques to confirm the time origin (Epp et al. 2012). eDNA in sediment samples is also sensitive enough to distinguish diverse communities that are geographically close (Laroche et al. 2020).

eDNA does not mix well in sediments, therefore it is necessary to take several subsamples that cover most of the sampling area. In general, subsamples between 0.25–1 g are collected for microorganisms, whereas 10–20 g are required for small invertebrates and macrofauna (Bruce et al. 2021). Samples are generally taken either with a sterile spatula (i.e., for shallow layers), a petite ponar grab sampler (Wildlife Supply Company) (i.e., for hard sediment samples), an epibenthic sledge (Glover et al. 2016), or a corer (Eichmiller et al. 2014; Inagaki et al. 2015; Glover et al. 2016; Bruce et al. 2021). Cores can be horizontally divided for further subsampling using single-use sterile syringes (60 ml) to extract minicores (Laroche et al. 2020; Capo et al. 2021). Clean tools between cores with 10% bleach and rinse them with double-distilled water (Laroche et al. 2020). Samples should be stored in Whirl-Pak bags on ice for up to eight hours and filtered within 24 h (Eichmiller et al. 2014). Samples should be preserved frozen at -20 °C, or -80 °C, or in some preservation solutions (e.g., ethanol for macrofauna, Qiagen's LifeGuard for microorganisms and meiofauna, and sucrose lysis buffer for extreme environments) (Mitchell and Takacs-Vesbach 2008; Bruce et al. 2021; Pawlowski et al. (2022). DESS can also be added for meiofaunal samples Pawlowski et al. (2022).

Protocols and laboratory pipelines for marine sediment can be found in the IODP/CDEX/KCC (2012) guideline and Glover et al. (2016). Furthermore, some of the field methodology aspects in Glover et al. (2016) are explained in a video (https://youtu.be/Io7NlFKUmYI). Pawlowski et al. (2020) have also developed sampling protocols for sediments, including step-by-step photographs of the whole sampling procedure. Pawlowski et al. (2022) provide a review regarding sediment sampling and DNA extraction. A guideline to choose the correct sampling and preservation method can also be found in Pawlowski et al. (2022: supplementary material). Capo et al. (2021) provide an overview and recommendations concerning sampling, extractions and THS technologies applied to sediments.

DNA can be extracted directly from the sediment sample, or the organisms (i.e., macrofauna) found in the sediment can be set apart by sieving and processed as a bulk sample (Bruce et al. 2021; Pawlowski et al. 2022).

Soil

Soil samples have been used for a long time to study taxonomic diversity in plants, fungi, and invertebrates (Thomsen and Willerslev 2015), as well as for characterising bacterial communities (Fløjgaard et al. 2019). Still two issues remain to be solved: the time persistence of eDNA in the soil matrix (Foucher et al. 2020), and the low representation of soil invertebrates in databases (i.e., BOLD) (Rota et al. 2020).

Ideally, two samples should be collected randomly or on a regular grid within each plot in the sampling location (Bienert et al. 2012; Taberlet et al. 2012). To account for soil heterogeneity, each sample has to comprise a pool of several (4–80) subsamples (Taberlet et al. 2012). The required amount of soil per subsample is usually 20 g, with a final volume ranging between 500 g to 4 kg (Taberlet et al. 2012; Clasen et al. 2020). Soil can be collected from the surface (a depth of 0–20 cm) and the sub-surface (a depth of 20–40 cm) using a soil corer, a disposable shovel, a thistle remover, a trowel, or by hammering a metal cylinder (Janzen et al. 2007; Bienert et al. 2012; Epp et al. 2012; Weinfurtner and Kördel 2012; Foucher et al. 2020). All tools should be sterilised with a high-temperature flame to avoid cross-contamination between soil samples (Bienert et al. 2012). Note that subsamples from different soil layers can be analysed independently to allow for comparisons (Rota et al. 2020) or can be pooled as suggested by Taberlet et al. (2012). Do not forget to remove the litter layer, large roots, and stones before sampling (Weinfurtner and Kördel 2012; Fløjgaard et al. 2019; Clasen et al. 2020). Soil samples can be placed into wide-neck barrels, Nalgene tubes or Whirl-Paks, and kept dark and cool at 4–5 °C until reaching the laboratory (Meyer et al. 2019; Clasen et al. 2020).

RECOMMENDATION
Design of soil sampling protocols depends on the diversity/habitat of targeted species and the location size. Refer to Taberlet et al. (2012) for further details. Furthermore, a pedological characterisation of the study area should also be done beforehand, as soil types and their microbial community will have different responses to storage methods (Schroeder et al. 2021).

Samples can be sieved (3 mm) to further remove stones and enable upcoming soil homogenisation (Clasen et al. 2020). Soil samples are generally processed immediately after collection (Taberlet 2012; Rota et al. 2020). If this is not possible, samples should be immediately freeze-dried, which is the best alternative for homogenising soils prior DNA extraction (Rüdel et al. 2011; Clasen et al. 2020). If impractical, samples can be placed in Ziploc bags containing silica gel bags for drying (Foucher et al. 2020), or they can be air dried at room temperature or using an unheated air source like a fan. Alternatively, DNA/RNA Shield (1 sample:9 buffer) or DESS solution (1 sample:3 buffer) can be added to the homogenised samples but note that these preservation solutions may induce some variation in the microbial taxonomic structure (Pavlovska et al. 2021). Do not pre-freeze samples, because of the risk of thawing, which can lead to spoilage and fungal growth (Clasen et al. 2020). Samples should then be stored at -20 °C or -80 °C for up to 13 months or until DNA extraction (Rhymes et al. 2021).

Airborne samples

Airborne eDNA studies have mainly focused on bacteria, fungal spores, algae, and pollen, with the key aim of monitoring airborne pathogens in relation to human health (Johnson et al. 2019; de Groot et al. 2021; Pumkaeo et al. 2021). Airborne eDNA methods have recently been applied to terrestrial ecosystems, proving its potential for population monitoring and biodiversity conservation (Johnson et al. 2019, 2021; Howell et al. 2021; Pumkaeo et al. 2021; Bohmann and Lynggaard 2022; Clare et al. 2022; Lynggaard et al. 2022a, 2022b). Both microorganisms and eDNA are scattered in the atmosphere, making their concentrations lower than in other environments (Banchi et al. 2020a). Sampling efficiency is, therefore, influenced by air volume, flow rate, collection medium, along with particle size and density (Behzad et al. 2015; Mainelis 2020). Several sampling approaches are used to collect airborne biological particles using either adhesive tape, air filters, or dust traps (Lynggaard et al. 2022a; see Mainelis 2020 for more details). Selecting the most fitting method depends on

the studied taxon. Note that the sampling procedure, i.e., type of chosen trap, and recovery procedure, and duration can overall affect the results, especially concerning the low represented taxa (Aguayo et al. 2018).

Air sampling protocols are not standardised, which makes it difficult to compare results from different studies to choose a method. Nonetheless, knowing the differences between the methods can help to choose the best option for your sampling design. Further research is also needed to improve bioaerosol sampling along with DNA extraction methods across taxonomic groups (Bohmann and Lynggaard 2022).

Gravity and passive sampling. This method relies on the ambient wind conditions at the study site (Johnson et al. 2019). It usually involves exposing Petri dishes containing agar (Mainelis 2020), or a mix of vaseline and paraffin wax to the outer atmosphere (Aguayo et al. 2018), or using Whatman paper filter (grade 1 or 3) (Mainelis 2020). The use of Tangle-Trap sticky coating on paper filters is also possible (Aguayo et al. 2018). These traps can be secured on blocks placed 1 m above ground for fungal spores and pollen collection (Aguayo et al. 2018). Note that this type of sampling may be affected by particle size and shape, preferring larger and heavier spores and pollen sorts (Behzad et al. 2015). There are four possible methods for recovering spores from paper filters: 1) by placing them in Petri dishes containing TE buffer, 2) rubbing, 3) grinding, or 4) shaking. Methodology details can be found in Aguayo et al. (2018). It is important to note that such sampling is the most sensitive to climatic conditions and hence, biological material may get lost (Aguayo et al. 2018).

Alternatively, Big Spring Number Eight (BSNE) dust traps can be used, mainly for plant community surveys (Johnson et al. 2019). To collect samples, each tray should be rinsed with 500 ml of deionized water, which should be pooled and collected into a single 1 l sterile bottle. Water samples should be labelled and transported dark and cool at 4 °C to the laboratory and filtered following the aforementioned water filtration method. Filters should be stored at -20 °C until DNA extraction (Johnson et al. 2019; 2021).

Active sampling. Vacuum or peristaltic pumps are utilised to actively collect particles, such as bacteria, spores, pollen, whole tiny arthopods and eDNA from animals (Ovaskainen et al. 2020; Clare et al. 2021; Serrao, et al. 2021). Sampling usually takes ca. 10 min. Samples can be collected either by using an adhesive coated tape (Melinex) placed on the sampler (Banchi et al. 2020b), or by directing the aerial particles into 1.5 ml sterile centrifuge tubes (Ovaskainen et al. 2020). After sampling, the coated tape can be stored at room temperature, and before processing, it should be cut into segments that can be rolled into 1.5 ml sterile centrifuge tubes, with the sticky side facing the internal part of the tube (Banchi et al. 2020b). On the other hand, the centrifuge tubes should be removed from the sampler and transported at room temperature. Final storage should be at -20 °C until further processing (Ovaskainen et al. 2020). If insects are found in the tubes, they should be rinsed with sterile water into the sampling tube and removed with sterile tweezers (Ovaskainen et al. 2020).

Active sampling also includes impaction sampling and filtration sampling.

Impaction sampling. Particles in the air are impacted onto a wet collection medium using either liquid impinger-based samplers (Behzad et al. 2015) or Andersen samplers (Mainelis 2020). Particles are collected into sterilised deionized water, NaCl solution or PBS solution with or without surfactants (e.g., 0–25% manucol), or onto Petri dishes with agar. Note that liquid media can evaporate within two hours, damaging the sample (Behzad et al. 2015). See Šantl-Temkiv et al. (2018) for detailed methodology. Samples should be kept at 4 °C.

Once a sample (mainly bacteria and fungi) is collected, the agar plates can be transferred directly to an incubator without intermediate steps, or they can be placed in Ziploc bags and placed into a Styrofoam cooler with cold packs, to be shipped off as soon as possible. Note that culturing methods, if considered, will leave out a considerable viable fraction of the total microorganisms that is not culturable.

RECOMMENDATION
The use of packed glass beads in the liquid impingers increases the efficiency of ultrafine bioaerosol (i.e., virus) collection. In addition, decreasing the sampling flow rate can increase the collection efficiency and reduce the loss of sampling liquid.

Filtration sampling. Filtration-based air samplers work by drawing air through filters of varying pore sizes, shapes, and compositions (Mainelis 2020). Capsule-enclosed filters with a pore size of 0.22 µm and 0.45 µm are commonly used (Clare et al. 2022). Once the particles are collected onto a filter, they can be eluted into liquid for subsequent analysis (Lynggaard et al. 2022a). Filters can also be placed dried in Ziploc bags and kept frozen until DNA extraction (Clare et al. 2022). Filter type, sampler type and speed will determine sampling duration. Sampling time ranges between 30 min and 30 h. This method has been successfully applied to terrestrial vertebrates (Clare et al. 2022; Lynggaard et al. 2022a).

Filters (e.g., F8 or F7 pleated fibrous particulate or HEPA), which are used for heating and ventilation systems, may also have the potential to collect eDNA as a byproduct of regular operation (Clare et al. 2022; Lynggaard et al. 2022a 2022b).

Paleontological/archaeological remains

Paleontological and archaeological collections provide us with access to ancient populations to determine their biology, anatomy, and surrounding environment. However, only samples from the Quaternary should be considered for biobanking and DNA analyses, as obtaining DNA from older samples is so far not possible.

Fossil samples can be discovered either by licensed excavations or by chance. Planned fossil collection includes three types of methods:

Dry sieving. Small fossils, such as teeth or bone fragments, can be found by using sieves of increasingly smaller mesh sizes. At the laboratory, fossils should be collected with the aid of a microscope (**Palaeontology Portal, American Museum of Natural History**, Seersholm et al. 2020).

Surface collecting. Bone fragments or isolated fossils can also be found on the surface of outcrops, without the need of digging holes larger than 1 m^2. All possible pieces should be collected, as they can be fragments of a larger bone, and should be wrapped in toilet paper and bagged. Field jackets made of plaster bandages might be necessary for larger specimens. (**Palaeontology Portal, American Museum of Natural History**).

Excavation. Excavating is a more demanding process, and some aspects should be considered:

1. Fossil position should be determined to ensure it is entirely collected.
2. Sites should be mapped, including elevation and orientation, by drawing and photographing.
3. Material used for DNA analyses should be lifted without using consolidants or other chemicals. Depending on the material, it can be packed in aluminium foil or Ziploc polyethylene bags. Bones should be left to dry in the field, then washed in water and dried before storing (Dabney et al. 2013). Specimens should always be handled with gloves.
4. Consolidants may be used *in situ* if bones are fragile. For instance, Paraloid B72 in acetone is used for "dry" excavations or polyvinyl acetate suspensions for waterlogged excavations. Note that minimal intervention is best, as some consolidant and adhesive methods are non-reversible. Refer to the **Florida Museum** for more details about consolidants.

5. Specimens may need to be coated with layers of aluminium foil, clingfilm, medical gauze immersed in plaster of Paris, or foaming polyurethane before lifting. Jacketing should be strong enough to protect the specimen during transport.

RECOMMENDATION
Do not forget to include information regarding stratigraphy, geography, taphonomy, and environmental factors to provide scientific value to the fossil.

Further protocols for sampling and recovering of bioarchaeological remains (e.g., faunal remains, plant fossils, charcoal material) can be found in Monk et al. (2007) and Campbell et al. (2011).

Permafrost-preserved samples

Permafrost-preserved bones, teeth/tusks, horns, skulls, and even large carcass remnants, like skeletons, and tissue belonging to extinct megafauna (e.g., steppe bison, woolly mammoth, woolly rhinoceros, horses, bears) are among the various fossils found in Eurasia and North America (Funck et al. 2020). Even logs and tree trunks have been found in Pleistocene peaty sediments (Martinez de la Torre et al. 2019). Usually, these fossils emerge during gold mining operations (Shapiro and Cooper 2003), construction activities (Zazula et al. 2017), or have been exposed and excavated from eroding riverbanks (Funck et al. 2020). Hence, sampling is considered opportunistic, and no excavation protocols are needed.

Gold miners regularly uncover fossils while thawing out frozen sediments to expose bedrock using high-pressure water and steam (Shapiro and Cooper 2003). Once the fossil has been removed from the permafrost, it will quickly deteriorate due to temperature and humidity fluctuations (**Florida Museum**). Fossil samples should not be kept frozen, unless they were recovered in that condition, because several freeze/thaw cycles damage DNA (Fulton and Shapiro 2019). Ideally, specimens should be dried slowly to avoid cracking and delamination (**Florida Museum**) and should be stored in a cool dry environment at a stable temperature (Fulton and Shapiro 2019).

RECOMMENDATION
Poor treatment after sample collection will be detrimental to DNA. Avoid heat, freeze-thaw cycles, and moisture.

A salvage excavation should begin when remains are found unexpectedly in a specific location (e.g., a construction site), to reveal the site stratigraphy and search for further remains. A backhoe should be employed to excavate slowly until a bone bed is detected, followed by a manual excavation using shovel and trowel (Zazula et al. 2017).

When permafrost carcasses are relatively complete, sediments within the body cavity should be discarded with a trench shovel before specimen removal from the site. Skin and any possible soft tissue should be removed from the bones by metal knives and scalpels. Hair, preserved soft tissue, and associated sediment should be individually stored and kept frozen (Funck et al. 2020). Samples for DNA studies should be taken before carcass chemical preservation (Maschenko et al. 2017).

Temperate-preserved samples

DNA preservation depends on the environment (Fulton and Shapiro 2019). Samples from mid-continental temperate areas tend to be poorer than those from high latitudes, as warm climates and humidity accelerate post-mortem degradation of DNA (Chang et al. 2017; Kehlmaier et al. 2017; Meyer et al. 2017). However, temperate, and humid tropical caves and water-filled sinkholes have been found to contain a high diversity of faunal remains where microclimate has been stable, and no harsh environmental changes have occurred since the Late Pleistocene (Slon

et al. 2022). These environments tend to be dark, cool, and wet, as well as anoxic and thermally buffered in the case of sinkholes, enhancing DNA survival (Dabney et al. 2013; Gutierrez-Garcia et al. 2014; Kehlmaier et al. 2017; Sheng et al. 2018). DNA has also been successfully retrieved from tropical lacustrine, swamp, and marine sediments (Kehlmaier et al. 2017; Dommain et al. 2020). Furthermore, dry sediments also seem to preserve DNA, although contamination and DNA leaching within stratigraphic layers may occur due to vertical transport (Dommain et al. 2020). Challenges of working with ancient samples from warm environments are described in Letts and Shapiro (2012).

Sedimentary ancient DNA (sedaDNA)

New methods for retrieving DNA from sediments are being developed to overcome the dependency on fossil remains, which are a limited and scarce genetic resource (Vernot et al. 2021). sedaDNA is absorbed by clay minerals, apatite, silica, or other sedimentary materials (Dommain et al. 2020). Further information on sedaDNA, including improvements and challenges, can be found in Capo et al. (2022).

The following procedure applies both to permafrost and temperate samples. Cores should be collected under controlled conditions and contaminations should be monitored (Willerslev et al. 2003). Before collection, core equipment should be washed with boiling water to reduce DNA contamination. Cores should be wrapped in plastic wrap and placed in acrylonitrile butadiene styrene (ABS) tubes for transport and permanent cold storage. Cores should be divided in two longitudinal parts. Ideally, one section should be kept as an archive sample, and the other one used for destructive subsampling. Sediment from the core surface should not be sampled to avoid any contamination (Dommain et al. 2020). Around 2 g of sediment should be taken from the core´s centre with sterile tools and stored frozen at -20 °C in falcon tubes until further analyses (Willerslev et al. 2003; Boessenkool et al. 2014).

Samples can also be collected either with a clean scalpel blade (Slon et al. 2022), a trowel, by inserting 50 ml Falcon tubes, or by using 5 ml screw cap tubes in a drill-like manner to excavate a few grams of sediment from the middle of each stratigraphic layer or from many points sampled every few centimetres of a pit sampling column. Usually, 1–2 mm of the surface material is removed to minimise contamination (Slon et al. 2022). Bulk-bone samples can be collected by excavating sediment and dry-sieving through 1.5–3 mm sieves (Seersholm et al. 2020). Samples should be placed in individual Ziploc plastic bags and stored in a refrigerator until further processing (Vernot et al. 2021). Sterile masks and gloves should be worn during sample collection to avoid contamination by modern DNA.

As aDNA concentration is lower in deeper strata, samples from cores or pit columns should be sampled from bottom to top. Note that the mentioned core subsampling procedure has to take place at a core storage facility rather than at an aDNA laboratory (Epp et al. 2019).

More recommendations regarding subsampling and decontamination of permafrost core samples can be found in Saidi-Mehrabad et al. (2020). For optimised sedaDNA extraction protocols from both frozen and non-frozen cores, refer to Haile (2012), Epp et al. (2019) and Vernot et al. (2021).

Palaeofaeces

Nonmineralized dung remains are called palaeofaeces and are usually found in caves and in dry areas. Palaeofaeces contain the DNA of both the defecator and the ingested organisms. Faeces should be cut into small pieces and added to the extraction buffer. Addition PTB, a thiazolium salt, into the buffer may help to improve the total DNA yield (Kuch and Poinar 2012).

Archaeobotanical remains

Archaeobotanical remains, mainly domesticated plant species suitable for DNA analyses, are scarce, not only because plant material tends to

decompose rapidly, but also because they are restricted to caves, arid areas, and waterlogged grounds. Most plant fossils are preserved by charring, and DNA basically cannot survive under such conditions (Nistelberger et al. 2016; Przelomska et al. 2020). Bunning et al. (2012) produced sequences from old, charred cereal grains. However, great caution should be taken when interpreting these data, as the obtained sequences might be artefacts of spurious mapping (Nistelberger et al. 2016). An assessment of carbon and nitrogen isotope values of archaeobotanical remains should be performed to determine the degree of charring before proceeding with DNA research (Nistelberger et al. 2016).

For protocols that can be used for non-charred desiccated and waterlogged plant remains see Gilbert et al. (2004), Wales et al. (2014) and Wales and Kistler (2019).

RECOMMENDATION
Phenol-chloroform extraction is preferred over commercial DNA extraction kits, which were found to perform poorly on botanical ancient samples (Wales et al. 2014).

Historical museum samples

Natural history museum collections function as banks for ancient and archival DNA. Museum specimens allow studying organisms for which fresh tissue samples are impossible to obtain, either because they are already extinct, or because they are only found in inaccessible regions. Furthermore, museum collections allow temporal comparisons between historical and modern populations. Obtaining endogenous DNA from museum specimens is feasible despite their unfavourable preservation conditions. A complete review regarding the advances and challenges in museomics is provided in Card et al. (2021) and Raxworthy and Smith (2021). Experimental and computational protocols to obtain genomic data from historical specimens can be found in Cong et al. (2021).

Museum and herbarium material also require special handling and processing comparable to palaeontological remains. The principles for aDNA should therefore be followed (see DNA chapter), as many museum samples yield low amounts of endogenous DNA (Kistler et al. 2020).

Be aware that the following aDNA methods apply both to palaeontological / archaeological material and museum material.

RECOMMENDATION
Benches should always be cleaned with bleach before extraction, then wiped clean with ultrapure distilled water. UV radiation can also be used to decontaminate benches. Forceps should also be cleaned with ethanol and flamed before handling specimens. Fresh blades and gloves should be used for each specimen.

Bones and teeth. Freshly excavated and untreated bones are preferred over fossil samples from natural history museums, as their preservation and storage conditions can be detrimental for DNA preservation. Fossils at museums are normally kept in cardboard boxes in rooms where both temperature and humidity are not stable. Hence, Pruvost (2007) suggested that fossils should be stored in a cold room, and ideally in a cryobank in small aliquots to avoid freezing and thawing cycles. Protocols at museums should be revised and assessed to improve preservation and retrieval of genetic data from archaeological/paleontological samples (Pruvost 2007; Seersholm et al. 2020).

Solid, heavy, and dense bones with few cracks are preferred for aDNA studies (Fulton and Shapiro 2019). The inner part of the petrous bone is considered the best source of mammal aDNA, and it has proven to be successful even with material from warmer climates such as Africa, the Near East, or Oceania (Hansen et al. 2017). For morphological identification in different taxa, see Bar-Oz et al. (2019). However, some destructive sampling is required, as the petrous bone has to be removed from the skull and the vestibulo-cochlear area has to be drilled into. Other options, such as the auditory ossicle and the circumferential lamellae of long

bones, have also been found to promote DNA preservation, due to the absence of bone remodelling (Ferrari et al. 2021).

Non-destructive pre-screening techniques should be applied before sampling begins to determine whether a bone is suitable for aDNA analysis (Keighley et al. 2021). The amount of organic matter, such as collagen, contained in fossil bones may indicate that DNA could also be preserved within them (Kehlmaier et al. 2017). Tripp et al. (2010, 2018) suggested a micro-computed tomography (MicroCT) method to identify microstructural features associated with collagen preservation, which may help to choose the best bone, and the best sampling location within the bone. Knowing how to select the right objects will reduce the destruction of zooarchaeological remains, helping with their long-term preservation (Ferrari et al. 2021).

Different from mammalian bones, teleost fish bones undergo bone remodelling to a lesser extent, or it is totally absent due to the lack of osteocytes (Shahar and Dean 2013). This improves the bone resistance to microbial degradation, making all archaeological bones, cranial, pectoral girdle or postcranial, suitable for aDNA studies, even if they look porous and brittle (Ferrari et al. 2021).

In general, no more than 1 cm^3 of bone or a single tooth root is used to recover DNA, although DNA quantity will decrease rapidly if the specimens are exposed (uncovered) for several seasons (Shapiro and Cooper 2003). The surface of the bone should first be removed using a Dremel rotary tool with a cutting blade. Engraving cutters and drills should be used to hollow out sections of the bone to gain access to the inner material (Fulton and Shapiro 2019). Bone powder can be collected by using either a Spex freezer mill or a 20 mm drill bit at very low speed (approximately 60 rpm) (Barnes et al. 2007). Note that a high drill speed will produce heat and vibration, which will be detrimental to DNA. The quantity of bone powder used for DNA extraction ranges between 30–200 mg following the Dabney et al. (2013) standard protocol that enhances the recovery of short aDNA fragments. For a more detailed protocol see Dabney and Meyer (2019).

Tooth root cementum is equally good as the petrous bone, in case it is not poorly preserved (no cementum, brittle, "chalk-like") (Hansen et al. 2017). Often entire tooth roots are lost when retrieving DNA because of drilling or grinding. New protocols use non-destructive sampling, and teeth can still be used for radio-dating or morphological studies. Check Hofreiter (2012), Gomes et al. (2015), and Harney et al. (2021) for further information. Tooth roots may be demineralized in 0.5 M EDTA (pH 8) in a thermomixer, set at 1000 rpm and 22 °C, for 24 h, prior to extraction (Eaton et al. 2015).

To remove extraction buffers after a non-destructive sampling method is performed, bones and teeth should be transferred to double-distilled water for 24 h at room temperature and the previous step should be repeated for another few hours. Samples should be removed from the tube and air-dried at room temperature (Hofreiter 2012).

Damgaard et al. (2015) recommended the following points when extracting aDNA from ancient bone or teeth:

- A brief predigestion step (15–30 min) should be applied, but only if more than 50 mg of the sample is available.
- the pre-digest supernatant should not be discarded at first, as it contains endogenous DNA. This also applies to undigested bone pellets post-24 h digestion.
- Several extractions should be performed if sufficient material is available.
- Tooth roots should be sampled from the surface rather than the inner dentine. The outermost surface layer should be removed before sampling to minimise the risk of contamination.
- The proportion of endogenous DNA will also be maximised if genome capture, single-stranded sequencing libraries, or damage-enriched single-stranded sequencing libraries are performed.

Note that a predigestion step with or without a bleach wash may not increase sequencing efficiency (Dehasque et al. 2022). Dehasque et al. (2022) recommend the use of MinElute

columns over QIAquick columns for ancient DNA extractions.

Hides. Skins should be cut into small pieces (5 × 5 mm) from the initial incision made during skin preparation using a sterilised surgical blade. This procedure should cause no significant loss and should not jeopardise further morphological studies (Moraes Barros and Morgante 2007). Each tissue sample should be transferred to a sterile Eppendorf tube. To diminish contamination, hairs and the outer layer should be removed with a sterilised scalpel and skin should be cleaned and washed three times with sterile milli-Q water, three times with 70% ethanol, then rinsed three times with sterile milli-Q water, and finally cut into small pieces. Museum skins were commonly treated with gasoline and butane, hence several washes with absolute ethanol for 24 h have to be performed to remove these PCR inhibitors (JEMU 2013).

Bird toe pads. This tissue is the best source of DNA from bird skins, as toe pads do not have much contact with preservatives (Lijtmaer et al. 2012). Approximately 2 × 4 mm of the inner toe pad should be removed with a disposable razor blade, avoiding the tissue surface. Tissue should be stored at room temperature until DNA extraction. Toe pads should ideally be rinsed in 0.5 M EDTA to wash away inhibitors, and DTT should be added in the first lysis step to reduce the amount of time required to completely dissolve the tissue. Detailed protocols can be found in Fulton et al. (2012) and Irestedt et al. (2022).

Keratin and chitin material. DNA can also be retrieved from hair, nails, horns, hooves, feathers, and insect cuticles, and these are simple to decontaminate (Campos and Gilbert 2019). Two crucial steps should be considered: 1) breaking the keratin/chitin by using detergents and reducing agents such as proteinase K, EDTA, and DTT; and 2) to purifying DNA using either silica-based methods or isopropanol when extracting with organic solvents (Campos and Gilbert 2019). Samples should be washed with a 0.5% commercial bleach solution and rinsed between two and six times in DNA-free water until traces of the bleach are removed (Gilbert et al. 2007a). A protocol for DNA extraction can be found in Campos and Gilbert (2019).

RECOMMENDATION
Add an extra silica purification step if DNA pellet is brown. Melanin is the cause of colouration and should be removed, as it may be a PCR inhibitor.

Insects. Non-destructive DNA extraction methods have been applied to pinned insects. In general, whole specimens should be placed in Eppendorf Biopur tubes, fully immersed in digestion buffer, and incubated overnight at 55 °C with gentle agitation for 16–20 h. Subsequently, specimens should be placed in 100% ethanol for 2–4 h to stop further digestion, air-dried, and returned to their collections. Refer to Gilbert et al. (2007b), Tin et al. (2014), Cong et al. (2021), and Cavill et al. (2021) for detailed protocols.

Eggs. Membranes from blown eggs can be removed through the blow-hole by using fine dissecting tools such as high-precision tweezers (e.g., no. s5/45 and 7), microforceps (no. 7) and fine shaped probes (T198 TAAB). This method preserves the egg´s appearance, patterning, colour, and shape dimensions, which is important for other scientific studies. Eggs with tiny holes and cracks around the blow-hole should be avoided. Samples should be stored in microfuge tubes at room temperature (Lee and Prys-Jones 2008).

DNA from fossil eggshells can also be obtained, as DNA is protected in calcite intracrystalline depositions within the eggshell matrix. A DNA extraction protocol can be found in Oskam and Bunce (2012). Note that the outer surface has to be removed either with a Dremel tool (>0.7-mm thin eggshell) or sandpaper (<0.7-mm thin eggshell) to avoid contamination.

Alcohol-fixed material. DNA in tissue preserved in alcohol at room temperature will degrade over time. However, different protocols have been developed to obtain useable DNA from fluid-preserved specimens, reviewed by Ruane (2021) and Bernstein and Ruane (2022).

Formalin-fixed material. DNA quality from formalin-fixed or paraffin-embedded tissues is affected by temperature, duration of exposure, and pH of the fixative. Campos and Gilbert (2012) suggested a DNA protocol that includes using heat and alkali treatment to break protein-DNA cross-links. A small piece of formalin-fixed tissue or thin slices from paraffin-embedded tissue can be taken using a sterile scalpel. Remove paraffin by trimming it away with a scalpel if it is present in large amounts. As heating is involved, O-ring screw-cap tubes should be used to avoid lids from being blown open. An optimised protocol for extracting RNA from formalin-fixed material can be found in Speer et al. (2022).

Resin-embedded specimens. It is under debate whether amber or sub-fossilised resins are suitable for genetic studies (Peris et al. 2020; Modi et al. 2021). DNA preservation in these materials may be limited by time and needs to be determined (Peris et al. 2020). A standardised protocol for insects embedded in resin has been developed by Peris et al. (2020), which can only be applied to modern samples (up to six years old). Although Modi et al. (2021) have proven that it is possible to recover endogenous ancient DNA from insects embedded in copal (a predecessor of amber), reproducibility of the results was not possible.

Mummified material. Ancient DNA research on mummified material, many animal species besides humans, is challenging, not only because mummification techniques, such as desiccation and anointment comprised the use of bandages and chemicals (e.g., heated oils, resins, natron) that are unfavourable to PCR conditions, but also because sometimes it is not anymore possible to verify how the original field collection occurred (Kurushima et al. 2012).

Another type of mummified material are the so-called bog men, i.e., human remains that survived in peatlands. Several types of tissue can be sampled by qualified staff following medical methods. Samples should be placed in vials containing distilled water and maintained in waterlogged conditions at 4 °C until further analyses are conducted (Mulhall 2020).

Samples should be removed using either a flexible fibre optic endoscope with grasping forceps, sterilised surgical tools or bore drill bits for larger animal mummies (Loreille et al. 2018; Hekkala et al. 2020). To prevent contamination with modern DNA, samples should be washed with 5–20% bleach for 15 min, then 80% alcohol, followed by rinsing with sterile water (Wasef et al. 2019). Muscle tissue (ca. 0.2 g) may be placed in glycine buffer for one week to three months with regular fluid changes to hydrate the sample (Hekkala et al. 2011). If drilling is necessary, the sampled area should first be wiped with 0.5% bleach and air-dried. Bone or tissue powder should be collected on a sterile foil reservoir (Hekkala et al. 2020).

For DNA extractions, follow Schwarz et al. (2009), Loreille et al. (2010), Rohland et al. (2010; 2018), Dabney et al. (2013), or Korlevic et al. (2015).

Herbarium material. Plant tissues preserved by freezing or desiccation are preferred for aDNA studies. aDNA has been successfully retrieved from seeds, wood, pollen, and vegetative tissues. However, a pure DNA extraction may be challenging because compounds, such as humic acids and polyphenols, along with heavily lignified epidermal layers, are considered PCR inhibitors (Wales et al. 2014). Additional reagents plus incubation time and temperature modifications to commercial kit protocols and removal of lignified tissue with a sterile blade should increase PCR success (Kistler 2012). Although DNA from herbarium tissue may be degraded, it is possible to obtain reliable sequence data (Staats et al. 2011).

Kistler (2012) suggested two protocols for DNA extraction: the CTAB protocol for single seeds, except for protein-rich, oily seeds (e.g., bottle gourd, sunflower); and the PTB (*N*-phenacylthiazolium bromide) protocol for gourd rind-like tissues and wood samples. See Dumolin-Lapegue (1999), Asif and Cannon (2005), Deguilloux et al. (2006) or Rachmayanti et al. (2006) for more wood DNA extraction protocols. Shepard (2017) suggested a non-destructive DNA sampling protocol for

leaves and stems, and Saługa (2020) for mosses. Cubero et al. (1999), Särkinen et al. (2012), Drábková (2014), Shepherd (2017) and Drábková et al. (2021) have also developed protocols for herbarium material.

A mortar and pestle or a mechanised mill can be used for grinding samples. Sterile sand can be added for tough samples. Small seeds should be ground in an incubation tube to avoid tissue loss. Consider incubating in buffer before grinding, to soften tough samples. Remember to sterilise the equipment with a bleach solution (10%) between samples (Kistler 2012).

Protocols for historical plant specimens can be found in (Marinček et al. 2022) and for historical lichen specimens and mushrooms in Dentinger et al. (2016) Forin et al. (2018), and Kistenich et al. (2019).

CHAPTER 4
Culture Preservation and Storage Methods

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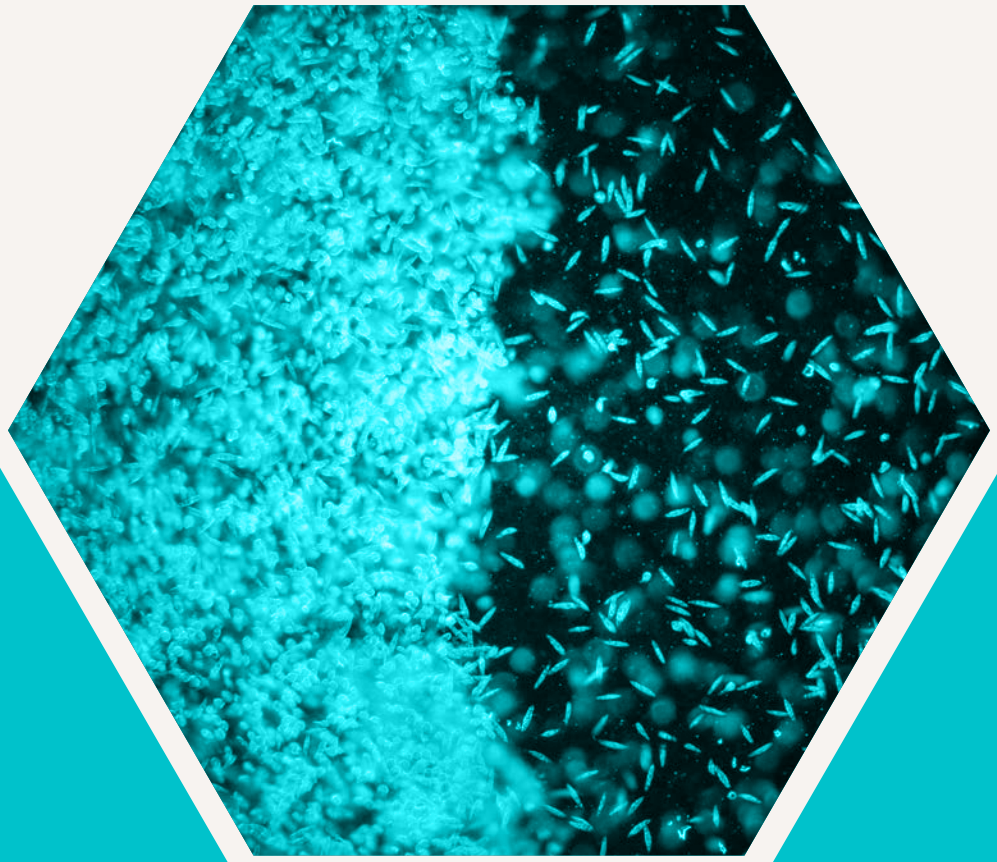

Protists and microfungi

Choosing the most appropriate preservation method is crucial to maintain the vitality, activity, immunogenicity, and genetic stability of microorganisms in living collections (Simões 2013). Most common preservation techniques are based on either maintaining continuous growth by subculturing or on induced dormancy by dehydration and freezing (Agarwal and Sharma 2006). Continuous culturing is suggested for short-term storage only (Ryan et al. 2000) since the approach is laborious and frequent recultivation may lead to contamination or sudden strain degeneration, which can cause morphology, physiology, or virulence to change (Vasas et al. 1998; Shivas et al. 2005; Bégaud et al. 2012; Homolka 2013). Furthermore, many microbial taxa are currently unculturable as suitable culturing conditions are unknown (Ryan et al. 2000; Ryan et al. 2019). Therefore, freeze-drying and cryopreservation at ultra-low temperatures are considered the best methods for long-term storage (Ryan et al. 2019).

RECOMMENDATION
Several strain/culture replicates should be preserved, following at least two preservation methods, commonly cryopreservation and lyophilisation, to lessen deterioration and risk of total loss.

Preservation of culturable microfungi, fungus-like organisms, microalgae, and other protists is well established, although recalcitrant species remain a challenge (Ryan et al. 2019). Whilst it is known that fungal spores can tolerate most conservation methods better than vegetative hyphae (Caleza et al. 2017), there is not yet a universal method that can be applied either to all fungal or all microalgal taxa (Ryan et al. 2000; Arguelles et al. 2020), and no method will assure indefinite and complete maintenance of a strain (Simões 2013). Ryan et al. (2000) developed a decision-based key to help determine the most suitable preservation method, considering not only the species, structure, and water content (Ryan and Smith 2004), but also the user facilities and the economic factor. The choice of a preservation method will also depend on the targeted storage period and the project's goals (Ryan and Smith 2004; Simões 2013).

Good cultural practices have been suggested by Crous (2002) and should be applied to both protists and fungi. Ryan and Smith (2004) and Arguelles et al. (2020) produced a table showing the advantages and disadvantages and also included storage time lengths possible with some of the methods mentioned below for fungi and microalgae respectively.

RECOMMENDATION
If permanent storage (e.g., LN2) is not possible, cultures should be regularly refreshed to keep viability and purity, as well as monitored by skilled systematists, who can check the success of preservation protocols, by assessing growth rate, morphology, viability, or molecular profiles.

Preservation on liquid and solid media

Serial or periodic transfers to new medium should take place every 3–6 months and cultures should be kept at 3–8 °C or at 15 °C if containing cold-sensitive strains (Shivas et al. 2005). Keep in mind that after many serial transfers, some strains may show a compromised ability to grow because of degeneration, which can ultimately lead to their loss (Heinonen-Tanski and Holopainen 1991). Fresh or herbarium-preserved mature specimens with spores or hyphae should be used for culturing and they should not have been previously refrigerated or frozen (Wrigley de Basanta and Estrada-Torres 2017). Culture flasks (e.g., McCartney) should be closed with a plastic membrane or another

means (e.g., plug or cap) to minimise contamination risks. As soon as a culture is established, several subcultures can be prepared. Some of these should be used for serial transfers at temperatures ranging from 4 °C to room temperature, whereas others should be allowed to dry or cryopreserved. Serial transfers can also be performed to maintain algal and cyanobacterial cultures under suboptimal conditions, but genetic and phenotypic stability cannot be ensured (Metting 1994; Friedl and Lorenz 2012).

RECOMMENDATION
Cultures should be inspected before subculturing to ensure the presence of viable cells. Subculturing procedures should be carried out in a laminar flow cabinet or a microbiology workbench in case of isolates producing airborne spores, and all necessary material (e.g., glassware, culture media) to be used should be sterilised. Bottle and test tube tops should be flamed over or otherwise sterilised before and after transfer (CCAP 2020).

Preservation can also take place on agar strips using vacuum-drying cultures, which may work for some groups otherwise difficult to preserve (e.g., *Pythium* (Oomycetes)) (Nakasone et al. 2004; Singh et al. 2018).

Pure colonies can be obtained after isolating one single spore or a hyphal tip. The former consists of serial spore suspensions until reaching an optimal dilution, which is then transferred to a Petri dish for incubation. The latter involves the removal of a hyphal tip from a mycelium edge and subsequent incubation to obtain a pure colony (Narayanasamy 2011; Capote et al. 2012). Many cultures can be stored at -20 °C or -80 °C in sterile 15–50% glycerol solution; the optimal combination will depend on the species (Akinsamni et al. 2017).

For permanent preservation as non-living specimens, non-sporulating cultures can also be dried in a laminar flow hood for two or three days. Then they are withdrawn from the Petri dish and placed in herbarium packets. Delicate fungi can be stored in small Petri dishes. To avoid curly and brittle results, the partially dried culture can be placed on hot glycerol agar and dried completely (Shivas et al. 2005).

Culture methods for slime moulds (Mycetozoa) have been described in Haskins and Wrigley de Basanta (2008) and in the "**Eumycetozoan project**". Note that it is highly difficult to keep axenic (i.e., contaminant-free) cultures of Myxomycetes, as they require bacteria as food source (Wrigley de Basanta and Estrada-Torres 2017). Mycetozoa cultures that are dried and stored at room temperature can be revived up to a year later by rewetting with sterile distilled water (Spiegel et al. 2004).

Generally, unicellular protist cultures should be established at conditions that resemble as much as possible their environment (light/darkness, temperature, oxygen, pH), and they should be maintained along the culturing process (Altermatt et al. 2015; Esteban et al. 2015). Initial cultures usually take place in microtiter plates and can be transferred to other containers such as culture tubes, Erlenmeyer jars with loose lids, or tissue-culture flasks for further subculturing. The culture medium will depend on the diet of the organisms (Esteban et al. 2015), but usually natural seawater/freshwater is used as a basis for growth media (Altermatt et al. 2015; Weber et al. 2017; Hansen et al. 2020). The use of a proteose peptone medium is recommended for axenic cultures, whereas a protozoa pellet medium is more suitable for non-axenic ones (Altermatt et al. 2015).

Organic material can also be added to the media, but this method is less standardised and unreliable (Lee and Soldo 1992; Altermatt et al. 2015), as some of the microorganisms will be unknown (agnotobiotic cultures). Nevertheless, most protozoan strains growing in liquid culture media need the addition of sterile cereal grains (CCAP 2020). Subculturing of strains should be done weekly or up to every two months, depending on the species. Note that every time a strain is subcultured, the food source (e.g., bacteria) will be also co-transferred (CCAP 2020). It is recommended to have 4–8 replicate cultures of each species stored in two separate incubators (Altermatt et al. 2015).

Original field samples of protists can also be used as culture, but they can only be maintained if the water sample contains a sufficient amount of plant material and is kept cool. However, this type of culture can only survive up to a week, at which point most species will have disappeared from the sample due to exclusion or predation (**Department of Biological Sciences**, George Washington University).

Protist soil samples can be cultured following the "non-flooded Petri dish method" to obtain ciliates (Foissner et al. 2002): Depending on the type of soil, ca. 150–600 g (including litter) are placed in a Petri dish, which is then over-saturated (110%) with distilled water and set aside one month. A species succession will occur, and therefore, examination should take place on predefined days (2, 7, 14, 21, and 28). Note that this method does not reactivate all cysts (Foissner et al. 2005). See Foissner et al. (2005) for a short description of other soil culture methods.

A comprehensive compendium of isolation and culture media for fungi, Oomycetes, and Myxomycetes has been produced by Bills and Foster (2004). For further preservation protocols and maintenance of fungal cultures including recommendations, refer to Singh (2017). Methods for cultivating microalgae can be found in Acreman (1994), Guillard and Morton (2004), Day et al. (2007), Garibay and Sadio (2020), and Martinez-Goss et al. (2020). Further culturing protocols for several protists can be found in Lee and Soldo (1992). Culturing protocols focusing on specific taxonomic groups are also available, i.e., for *Acanthamoeba* species (Axelsson-Olsson et al. 2009), choanoflagellates (King et al. 2009) and ciliates (Liu et al. 2019). Media recipes for protists can be found in Andersen (2005) and Altermatt (2015).

Preservation in mineral oil

This method is ideal for mycelial and non-sporulating cultures that cannot undergo freezing, except Basidiomycota (Homolka 2014). Sterile mineral oil is used to cover agar cultures, thus preventing dehydration and slowing down metabolic activity and growth (Bégaud et al. 2012; Caleza et al. 2017; Singh et al. 2018). Vials can be sealed with parafilm before storage (Abd-Elsalam et al. 2010). Even though this method reduces mite infestations, vials need to be regularly inspected for air-borne spore contamination (Homolka 2014). Cultures can persist for up to 40 years, depending on the species, and ideally renewal should take place every five years (Shivas et al. 2005). This method is preferred when lyophilisation or cryopreservation is not possible (Ryan et al. 2000). Note that selection of mutants may occur due to fungi continuously growing in this preservation method (Homolka 2014). Singh et al. (2018) provide a protocol for long-term preservation of live cultures.

RECOMMENDATION
Cultures should be immediately examined and discarded if a mite infestation occurs. Cultures that must be saved can be frozen at -20 °C to kill the mites and eggs.

Preservation in water

Young and vigorous colonies, including spores, hyphae, or yeasts, on agar are cut in blocks and placed in McCartney bottles, containing sterile distilled water. The bottle lids should be tightly screwed down. Storage is recommended at room temperature (Agarwal and Sharma 2006) or kept cool in a refrigerator (Abd-Elsalam et al. 2010). Water and inoculum blocks should follow a 40:1 volume ratio. If excessive amounts of inoculum and medium are added, the fungus will not endure (Caleza et al. 2017).

This method works very well for early-diverging fungi, filamentous fungi, moulds, and yeasts which can survive more than a decade, including plant pathogens (e.g., wood-decomposing Basidiomycota and oomycetes such as *Pythium*, *Phytophthora* spp.), and ectomycorrhizal fungi (Heinonen-Tanski and Holopainen 1991; Burdsall and Dorwoth 1994; Bégaud et al. 2012; Homolka 2013). This method is not recommended for taxa previously assigned to Zygomycota since these are less stable in this type of preservation (Singh et al. 2018).

Preservation in soil

This method is ideal for microorganisms that can produce conidia (asexual spores) or resting structures (Jong and Mirmingham 2001; Bégaud et al. 2012). A spore suspension is added to sterilised sieved soil and incubated at room temperature for 5–10 days depending on the fungus growth rate. Then it should be stored at 4–7 °C (Shivas et al. 2005; Agarwal and Sharma 2006). A detailed protocol can be found in Singh et al. (2018). Soil-borne pathogens (e.g., *Fusarium*) can be preserved by this method (Abd-Elsalam et al. 2010); however, soil preservation is not recommended for ectomycorrhizal fungi (Heinonen-Tanski and Holopainen 1991).

Preservation in silica gel

Silica gel can prevent fungal growth and metabolic activity, minimising the risks of genetic and morphological changes (Abd-Elsalam et al. 2010). Some fungi can be stored for up to 18 years in this way (Bégaud et al. 2012). Typically, glass flasks are filled to one quarter with non-indicating silica gel and sterilised in the oven at 180 °C for 3h. Bottles should be placed in a water tray and frozen in a deep freezer. Spore suspensions are added to the bottles and incubated at 25 °C for 10–14 days. Bottles are then stored at 4 °C in airtight containers with indicator silica gel. Silica gel is suitable for organisms that produce sclerotia or chlamydospores (Shivas et al. 2005). However, this method is not ideal for thin-walled spores, spores with appendages and mycelia, or for ectomycorrhizal fungi (Heinonen-Tanski and Holopainen 1991; Agarwal and Sharma 2006). Two protocols are provided in Nakasone et al. (2004).

Preservation on wood chips

This method has been designed specifically for wood-inhabiting fungi (e.g., Basidiomycota and Ascomycota), but some pathogenic fungus-like protists can also be maintained with this preservation method (Abd-Elsalam et al. 2010). Around 50 small pieces of wood should be collected into a flask, which should be sterilised at 121 °C for 20 min twice. Fifteen chips should be placed on pre-grown colony Petri dishes and incubated for 10–15 days. Colonised wood chips are transferred to sterile test tubes, which are stored at 4 °C (Nakasone et al. 2004; Singh et al. 2018).

Collected pieces of rotting wood can also be placed in standing water in a Petri dish or onto agar plates to grow protists such as protostelids. Cultures should be kept in moist chambers and inspected daily for up to two weeks with a microscope (Spiegel et al. 2004).

Preservation on sterile cereal grains

This method uses sterile cereal grains (e.g., rye, barley, millet) to grow both microfungi (including pathogenic species, e.g., *Rhizoctonia*), and mushrooms (see macrofungi section below for more details). After inoculation, microfungi can be stored at -20 °C to 5 °C, depending on the species. It should be used for storage times of less than one year (Singh et al. 2018). One advantage of this method is that subculturing is not needed (Singh 2017). Cultures can be dried in a desiccation chamber and kept at -25 °C. Alternatively, colonised grains can be transferred to a sterile Eppendorf tube and stored at -80 °C (Abd-Elsalam et al. 2010).

Preservation on sterilised filter papers

Agar plugs are placed on the filter paper (e.g., Whatman No. 1) and stored in Petri dishes at room temperature until the paper is fully colonised by fungi. Filter paper should be completely dried, cut in small pieces and stored in air-tight vials at 4 °C (Shivas et al. 2005). A protocol can be found in Singh et al. (2018). Mycorrhizal fungi and saprotrophic

fungi can be preserved on charcoal filter paper strips for subsequent cryopreservation (Stielow et al. 2012). Ciliate dried cysts can also be preserved using filter paper (McGrath et al. 1977).

Preservation on cotton cloth

Microalgae can be immobilised and preserved in pieces of cotton cloth at 4 °C in the dark. Refer to Prasad et al. (2016) for details.

Note ectomycorrhizal fungi and obligate pathogens can only be maintained with their host plant either in diaxenic or semi-aseptic cultures.

Microbiomes

A substantial number of protocols is available for pure culture preservation and storage of microorganisms, but techniques are still scarce for intact (unhomogenised) samples or microbiome preservation. Some techniques developed in the food industry and therapeutics can be applied to the long-term preservation of microbiomes, such as CAS (cell alive system) freezing under an alternating magnetic field (Morono et al. 2015) for up to six months, or high-voltage microencapsulation techniques (e.g., electro-spraying and electro-spinning) (Lim 2015; Rodríguez-Tobías et al. 2019; Stojanov and Berlec 2020). Prakash et al. (2020) provide an overview of current preservation methods of intact samples, mixed cultures and microbiomes.

Macrofungi

For long-term storage, dried mushroom specimens are usually kept in herbarium boxes or packets (Lodge et al. 2004). If the main aim is to culture macrofungi, they should be transported to the laboratory as quickly as possible. Otherwise, a cultivation chamber should be taken into the field. Tissue from the sporophore should be taken from the junction between the cap and the stipe. The tissue should be precleaned before inoculation, then broken to expose the inner mycelium, which should be cut into small pieces (ca. 1–3 mm^3) with a sterile scalpel. Avoid any contact with the outer surface to reduce the risk of contamination. Pieces should be placed on Petri dishes containing agarised medium, or test tube slants for cultivation (Heinonen-Tanski and Holopainen 1991; Vasas et al. 1998; Lodge et al. 2004).

Mushrooms can be preserved on wooden chips placed on agar media (Lodge et al. 2004). Mycelia (e.g., *Agaricus* spp.) can also grow on cereal grains or straw (i.e., solid-substrate cultivation), and as soon as these substrata are colonised, they can be transferred or placed in sterile tubes for storage (Sánchez 2010). Alternatively, mycelia from Basidiomycota can be grown in a liquid medium (submerge cultivation) by using a shaker or a bioreactor system (Bakratsas et al. 2021). A troubleshooting list for mushroom cultivation is provided in Sánchez (2010).

Basidiospores and ascospores can also be used to start cultures, by letting the mushroom shoot its spores directly onto the agar surface. For some fungi, it is necessary to add organic matter from their original substratum for them to grow. Note that spores from tropical fungi must be cultured right after collection, since they lose viability comparatively quickly and are sensitive to desiccation. Spores can also be obtained from spore prints (i.e., print of the fungal lamellae on paper or glass used for fungal identification) and can be cultured using a streaking method (Lodge et al. 2004).

Lichens

For permanent storage, lichen specimens should be placed in acid-free paper with 25% rag content and stored at room temperature (Buyck et al. 2010). They can, however, still deteriorate with time and green lichens may lose their colouration (Honegger 2003). Ideally, lichen thalli and spores ejected from apothecia can be stored dried in vials at -20 °C without losing their macro- and microscopic features (Honegger 2003; Zakeri et al. 2022).

Lichens are very difficult to culture due to their symbiotic nature. Thallus fragments are generally used for *in vitro* techniques (Yamamoto et al. 2002), but spores can also be used to start cultures (Zakeri et al. 2022). If apothecia do not release spores or if the lichen species does not regularly produce apothecia, it will not be possible to culture spores (Yamamoto et al. 2002). It is necessary to first separate the lichen symbionts, the mycobiont and the photobiont, and keep them in axenic conditions (Yoshimura et al. 2002). The isolation of the mycobiont is more problematic since it is a slow and long process with a high contamination probability (Zakeri et al. 2022). Pichler et al. (2021) established protocols to enhance culture conditions for mycobionts. Ideally, both axenic symbionts should be again associated to reform the lichen thalli (Yoshimura et al. 2002). Unfortunately, there are not many examples of cases where this has been successfully achieved (Stocker-Wörgötter 2002). Thallus fragments from foliose lichens can also be cultured outdoors, following Haggener (1993).

Protocols for ascospore and conidia discharge, isolation from soredia and thallus fragments, and cultivation procedures, can be found in Zakeri et al. (2022) and references therein. Černajová & Škaloud (2020) also developed a protocol to culture lichen soredia. For further information see Verma and Behera (2015), where tissue culture of various groups of lichens is reviewed.

Macroalgae

Tissue pieces (ca. 4 cm^2) of macroalgae should be stored dried, using silica gel, which can subsequently be used for molecular studies (Azevedo Neto et al. 2020). By selecting clean apical parts of the thallus, or by wiping the thallus piece with clean tissue, co-sampling of epiphytes can be avoided. The rest of the thallus should be kept as an herbarium specimen (Heesch et al. 2008). For anatomical examination and study of reproductive cells, it can be useful to store a (fertile) piece of the thallus in 70% ethanol or a solution of 4–5% formaldehyde in seawater. Further description of the processing and storage of seaweeds for commercial uses can be found in GENIALG (2017).

The choice of tissue to be cultured will depend on the seaweed group or species (Wade et al. 2020). Isolation and culturing from young vegetative tissue is typically done for species with apical meristematic growth (Kawai et al. 2005). To remove epiphytes, the tissue piece is cleaned with a clean paper towel. The apical portion should be cut and placed into a multiwell plate containing sterilised seawater to remove further epiphytes following the dilution method (see "field collection" chapter, protist section). If the apex is big, it should be vortexed under addition of seawater (Kawai et al. 2005). Cultures can be preserved as meristems or mature thalli (Wade et al. 2020). Alternatively, between two to four fertile thallus pieces can be selected from each individual sporophyte, which should be dried with paper towels, wrapped in aluminium foil, and kept at a cool temperature (e.g., 10 °C) overnight to provide early sporulation. Sori should be removed with

a hole punch and placed in Erlenmeyer flasks with sterilised filtered seawater. After spores are released, they can be cultured in Petri dishes until they turn into gametophytes (Barrento et al. 2016). It is important that the gametophytes are maintained in a vegetative state (Bartsch 2018). Further details for gametophyte clonal stock culture procedures are found in Bartsch (2018). Detailed isolation and culturing protocols for vegetative cells and spores are provided in Kawai et al. (2005). Culture conditions and equipment can be found in West (2005) and Campbell and Field (2020). It is strongly recommended to maintain cultures under dormancy conditions for better preservation (Wade et al. 2020).

RECOMMENDATION
Prior to subculturing, seaweed culture flasks should be examined by eye or under a stereoscopic microscope to ensure the presence of motile and very dense cells, which are suitable for inoculation (CCAP 2020).

Plants

Plant material can be conserved via their generative and vegetative organs or tissues. Depending on the purpose, different preservation strategies are used. For the conservation of plant genetic resources, most angio- and gymnosperm species are stored as orthodox, desiccation tolerant seed in seed banks. Spores or gametophyte cultures of bryophytes and pteridophytes, or seed plant species species that do not breed true or develop recalcitrant, desiccation-sensitive seeds, or have no seed production whatsoever are all maintained under slow-growth conditions in tissue culture, cryopreserved or often grown in field genebanks (Saad and Rao 2001). In some cases, especially for wild seed plant species, pollen is used to conserve the haplotype. Plant tissue can be stored dried, lyophilised, or frozen under different temperatures.

Storage under dry and low temperature conditions

Pollen. Comparable to seeds, pollen can also be tolerant or sensitive to desiccation. Most pollen grains are desiccation-tolerant and have moisture contents below 30% (fresh weight basis) at dispersal. They are also termed partially dehydrated or orthodox and are characterised by small size, often between 30 µm and 100 µm (Pacini and Franchi 2020) and a storability at low moisture contents (< 15%) and low temperatures (Dinato et al. 2020). Pollen grains with a moisture content of above 30% at dispersal are often desiccation-sensitive and are also termed partially hydrated or recalcitrant. They often measure between 15 µm and 150 µm (Pacini and Franchi 2020) and commonly do not tolerate water loss below 30% (Nebot et al. 2021). However, pollen of species producing orthodox seeds are not necessarily desiccation-tolerant (Franchi et al. 2011).

Orthodox pollen can be stored dry in Eppendorf tubes, cryovials, glassine bags or gelatine capsules sealed airtight in aluminium pouches at -20 °C, -80 °C or -196 °C in LN2 (Anushma et al. 2018; Sidhu 2019). Drying is recommended at RH near 30% (Nebot et al. 2021). Alternatively, pollen may be stored dry at 4 °C, but viability will decrease after a couple of days or weeks, depending on the species (Sidhu 2019). Pollen is generally very short-lived, and viability can eventually decrease even during LN2 storage (Pence et al. 2020), for yet unknown reasons. Therefore, viability testing procedures should be standardised and carried out periodically (Towill and Walters 2000).

Recalcitrant pollen requires cryopreservation (see cryopreservation chapter). Note that the moisture content has to be chosen carefully. It needs to be above a level at which desiccation damage occurs and below or close to the limit at which water can freeze (Nebot et al. 2021).

Seeds. More than 90% of angiosperm species are known to produce orthodox seeds that can tolerate water contents of less than 10% (Alpert 2005; Hoekstra 2005). Hence, they can be maintained for extended periods if storage conditions are optimal, namely dry and cool (FAO 2014). By contrast, less than 10% of the species are known to produce recalcitrant or intermediate seeds that do not survive drying or are sensitive to drying, respectively (Wyse et al. 2018). However, so far, many seeds of tropical species have not been classified yet and might belong to the last two categories. In any case, seed storability is dependent on seed moisture content, storage temperature and atmosphere (Roberts 1961, 1972; Theilade and Petri 2003). Anoxic conditions may prolong seed longevity during storage (Groot et al. 2015). A compendium of storage behaviour of different species and protocols to determine seed storage behaviour can be found in Hong et al. (1996), Royal Botanic Gardens Kew (2017), and **MSBP-Kew technical information sheet 10**. Further information about factors that affect long-term conservation of seeds in genebanks are provided by Kameswara Rao et al. (2017), and suggestions to improve seed longevity can be found in Ballesteros et al. (2020). Comprehensive overviews regarding seed bank storage can be found in Smith et al. (2003), Hay and Probert (2013), Solberg et al. (2020) and Martyn Yenson et al. (2021).

Orthodox seed storage. Typically, dry mature seeds are further dried to an equilibrium moisture content in a controlled environment of 5–20 °C and 10–25% RH. Long-term storage collections are mainly kept at -18±3 °C (FAO 2014; MSBP 2015). Note that seeds of wild relatives may behave differently from seeds of domesticates, hence optimal storage conditions should be individually defined (Engels and Visser 2003). For optimal short-term storage, the seeds should be stored at the same temperature at which they were dried, which is usually 15 °C and 15% RH (ENSCONET 2009; FAO 2014).

Silica gel can be useful for drying seed collections to recommended moisture contents in the laboratory, in the field, or at other situations where a permanent drying facility is not available. However, it is advisable to monitor the seed moisture content when seeds are kept in closed containers with indicating (colour-changing) silica gel for extended periods. There is a lower limit for the effectiveness of moisture content reduction in extending storage longevity and for some otherwise orthodox seeded species (e.g., *Salix* spp.), over-drying can be harmful (Walters and Engels 1998; John Dickie pers. comm.).

Some species produce orthodox seeds that are short-lived, such as seeds from *Allium* species or lettuce (Nagel and Börner 2010), or various wild species. These species are prepared for storage rapidly after maturation drying or may be stored in the vapour phase of LN2 (see cryopreservation chapter).

Alternatives to silica gel drying are ultra-dry storage (Hong et al. 2005), sun/shade drying (Hay and Probert 1995, 2013), and vacuum/freeze drying that allow storage at room temperature (Theilade and Petri 2003). The use of zeolite beads is common in horticulture, allowing seeds to be kept at room temperature (Hay et al. 2012; **University of California, Davis**). The **protocol** for seed drying and storage using zeolite beads has been developed at the University of California Davis.

Recalcitrant and intermediate seed storage. Unlike orthodox seeds, recalcitrant seeds are not desiccation-tolerant and die when exposed to low RH and lower temperatures, whereas intermediate seeds usually have short lifespans in dehydrated or/and low-temperature conditions (FAO 2014; Ballesteros et al. 2021). For short- or medium-term storage (i.e., in the case of tropical species or in some temperate species), recalcitrant seeds are maintained in hydrated storage to avoid water loss, which can lead to germination or poor viability and vigour (FAO 2014). Seeds should be placed into paper bags within polyethylene bags, or they can be placed in sterilised plastic buckets with seal-

ing lids. In general, seeds should be stored at the lowest temperature they can tolerate, and it is critical that temperature remain constant (i.e., temperate seeds 4 ± 4 °C, tropical seeds 16 ± 2 °C). Containers should be regularly ventilated to evade anoxic conditions (FAO 2014).

RECOMMENDATION
Be aware of fungal proliferations, which are usually a consequence of hydrated storage. These can considerably affect the lifespan of the embryonic axis. Use a selection of fungicides to eliminate fungi. Otherwise consider seed germination as an alternative to hydrate storage.

Plant tissue can be stored in various ways: 1) boxes containing small amounts of silica gel and a relative humidity indicator card, 2) into vials containing RNAlater or a similar product, 3) in CTAB buffer at -20 °C, or -80 °C, or 4) they can be flash-frozen in liquid nitrogen, particularly if high molecular weight DNA is required (CPC 2019; Forrest et al. unpublished)

In vitro tissue culture techniques

In vitro techniques are important tools for conservation, especially for the propagation, regeneration, multiplication, and restoration of plant genetic resources that cannot be efficiently maintained and regenerated via seeds (Rowntree 2006). Vegetatively conserved species are mainly those that rarely or never produce seeds (e.g., garlic, banana), species with recalcitrant or intermediate seeds, (e.g., mango, coffee, papaya), or species that do not breed true and produce heterozygous seeds (e.g., potato, apple, strawberry) (Reed et al. 2013). Commonly, these species are immediately introduced into tissue culture (Pence et al. 2002) and preserved under so-called 'slow-growth conditions' for short- and medium-term storage (Chauhan et al. 2019; Panis et al. 2020). Benefits and risks of plant tissue culturing are described in Sommerville et al. (2021).

RECOMMENDATION
Data regarding morphological variation, treatments used (e.g., growth conditions, hormones, medium) as well as the environmental conditions (light, temperature, gas atmosphere) should be collected at all stages of propagation to use as base information of the plant response to *in vitro* procedures.

It is important to note that *in vitro* tissue culture should be initiated immediately after plant material collection, either in the field (see plant collection section) or more commonly in the laboratory. For introduction to tissue culture and propagation of *in vitro* plantlets, young or growing tissues, preferably meristematic tissues, are used as explants. This often includes shoot tips, nodal and floral segments, embryos, and embryonic axes (Paunescu 2009). Explants are commonly immersed in sodium dichloroisocyanurate (NaDCC or SDICN) or sodium hypochlorite to limit endophytic contamination prior to placing them onto an axenic culture (Engelmann 1997; Sarasan et al. 2006; Senula and Nagel 2021). Possible solutions to overcome critical obstacles (e.g., tissue and medium browning or necrosis) to successful culture initiation include the addition of activated charcoal, DMSO or PVP, or transferring to fresh media cultures (Sarasan et al. 2006). A detailed review regarding contamination control during *in vitro* initiation can be found in Pence et al. (2002) and Reed et al. (2004).

RECOMMENDATION
Check regularly for contamination, plant degradation and browning during every step of the *in vitro* cultivation. If needed, transfer to new media.

In general, tissue culture protocols and media composition should be developed for each plant group, species, or in some cases even for specific genotypes, as they can behave differently in similar culture conditions (Ashmore

1997). Before culture initiation, it should be determined whether different media for initiation, proliferation and storage are necessary (Reed et al. 2004). In addition, growth conditions (e.g., light quality, intensity, and exposition time, temperature, O_2 and CO_2 concentration, availability of space, and addition of mycorrhiza) should be carefully adjusted and controlled for different species (Kubota 2001; Sarasan 2010), especially for those that require particular treatments to grow *in vitro* (e.g., immature seeds from temperate orchids can only germinate using a mycorrhiza-assisted germination technique (Diantina et al. 2020)). Depending on the species, cultures may be maintained in liquid or solid media and can contain macro- and micronutrients, sugars, supplements such as phloroglucinol or difluoromethylornithine (DFMO), and phytohormones to promote regeneration (Sarasan et al. 2006; Chauhan et al. 2019; Panis. et al. 2020).

RECOMMENDATION
When species are recalcitrant in culture or when little material is available, tissue culture approaches should be carefully considered.

In recent decades, different techniques have been widely implemented for conservation, propagation, and multiplication of plant material, mainly of Angiosperms (flowering plants) and Gymnosperms (conifers, cycads). *In vitro* techniques are dependent on the plant species, the intended use and the targeted organ (Reed et al. 2004; Sarasan et al. 2006; Cruz-Cruz et al. 2013; Sommerville et al. 2021):

- **Micropropagation of nodal segments, axillary or terminal vegetative buds, or excised meristems.** Generally, plant segments containing meristematic tissue are used for vegetative propagation (Engelmann 2010). There are a number of protocols established for root and tuber crops, fruit trees, ornamental and medicinal plants, and wild species among others, from temperate and tropical origin (Pence et al. 2002). For root cuttings of woody species, vermiculite, coir, perlite, or paper pulp can support efficient root development (Sarasan 2010).
- **Callus culture.** Callus cells are considered unorganised, non-differentiated meristematic cells that can develop into a differentiated plant under appropriate conditions (Efferth 2019). To reduce the risk of mutations, callus cultures are usually not used for the conservation of plant genetic resources (Panis et al. 2020). However, it has been applied efficiently for mass production of plantlets (i.e., in tree species such as *Neolamarckia cadamba*) (Huang et al. 2020). Different ratios of plant growth regulators of auxins, cytokinins and gibberellins are used to stimulate callus cultures or root and shoot formation (Efferth 2019).
- **Suspension cell culture.** Loose clumps of callus are usually placed on a liquid culture medium, where they break into small fragments to form a cell suspension. Usually, a sample of this solution is transferred to other types of medium to induce the production of more callus, somatic embryos, or adventitious shoots (Sommerville et al. 2021).
- **Somatic embryogenesis.** In somatic embryogenesis, the formation of plant embryos from somatic cells is initiated. This can occur in nature under stressful environmental conditions. In tissue culture, it is controlled by plant growth regulators and includes induction, embryo development, maturation, and germination (Méndez-Hernández et al. 2019). The composition of the media and environmental conditions are species-dependent, and require precise adaptation (Bhojwani and Dantu 2013).
- **Protocorm-like bodies (PLBs) formation**. PLBs are a specific type of somatic embryos that occur only in orchids and are highly used for micropropagation purposes (Cardoso et al. 2020). Reviews, including optimised parameters for the development or improvement of protocols, can be found in Teixeira da Silva et al. (2015), Zanello and Cardoso (2019) and Cardoso et al. (2020).
- **Zygotic embryo culture.** Zygotic embryo culture is often implemented after so-called

"embryo rescue", which implies the excision of embryos from seeds to assist the development of a vigorous plant from dormant, weak, immature, or hybrid seeds (Raghavan 2003). Culture methods are reviewed in Winkelmann et al. (2010) and Thorpe and Yeung (2011).

- ***In vitro* seed germination.** To support seed germination, seeds are disinfected and grown under optimum conditions on agar. After normal seedlings develop, they are commonly used for molecular studies or transferred and planted into soil.

RECOMMENDATION
Valuable and irreplaceable *in vitro* plants should be handled carefully. In case of contaminations, transfer onto new media or introduction to soil and greenhouse conditions need to be considered.

For bryophyte tissue growth, cultures are commonly started from spores but sporophytic and gametophytic tissues can also be used to initiate cultures. Plant material should be sterilised using NaDCC (sodium dichloroisocyanurate) without further detergents, as they can have detrimental effects on the cultures. Ideally a pre-culture step should take place to increase the amount of starting material before culture initiation. Two protocols can be found in Rowntree (2006).

RECOMMENDATION
Always confirm that the produced gametophytes and sporophytes belong to the target species, as foreign spores traveling by wind may also have been included during the collection procedure.

For fern tissue growth, spores are sterilised to generate *in vitro* gametophyte cultures and sporophyte culture lines. Sporophyte cultures can also be initiated using other fern tissues such as rhizomes, shoot tips or bud scales. However, the culture growth often takes weeks to years, until a sporophyte can be obtained. Refer to Ballesteros and Pence (2018), Barnicoat et al. (2011) and Menéndez et al. (2011) for detailed protocols and suggested media.

RECOMMENDATION
All resulting material from in vitro processes can be used for cryopreservation.

Storage of in vitro *cultures*

Plants are commonly kept in *in vitro* culture (or tissue culture) for large-scale micro-propagation, genetic manipulations, and somatic embryogenesis. Thus, plant material is kept alive under sterile and well-defined conditions including required nutrients, plant growth regulators and water (Panis et al. 2020).

Slow-growth preservation

Slow-growth preservation procedures are suitable for short-term and medium-term storage (Chauhan et al. 2019). This approach reduces staff time because subculture intervals can be prolonged to up to four years (Ashmore 1997). Various factors should be considered when using slow-growth procedures and a combination of these is commonly used for several species (Ashmore 1997; Reed et al. 2004; Sarasan et al. 2006; Keller et al. 2011, 2012; Priyanka et al. 2021; Senula and Nagel 2021; Sommerville et al. 2021):

- Low temperature: plant material is kept at temperatures ranging from 1° C to 10° C for cold-tolerant species, and from 15°C to 28°C for tropical species.
- Reduced light conditions or culture in the dark.
- Addition of osmotic agents (e.g., mannitol, sorbitol, PEG) or growth retardants (e.g., abscisic acid). Be aware that these may increase somaclonal variation. Culture media should be preferably hormone-free.

- Covering explants with mineral oil or paraffin can be useful to reduce culture growth (Cruz-Cruz et al. 2013).
- Low oxygen: the concentration of oxygen is reduced, but the atmospheric pressure is maintained by adding nitrogen. Plant tissue growth will decrease, as will the photosynthetic activity.

RECOMMENDATION
Maintaining plants *in vitro* for prolonged periods of time can lead to epigenetic changes and somaclonal variations. Flow cytometry, phenotype analysis or the use of molecular markers can help to detect somaclonal variations. Ideally, *in vitro* media and conditions should be adapted to keep genetic integrity of the cultures.

Synthetic seed technology

Plant propagules, especially somatic embryos, nodal segments, shoot tips, and somatic embryos, as well as spores are artificially encapsulated in a protective coating, forming the so-called synthetic seed (Jang et al. 2020). Seed production and storage can be accomplished either by slowly desiccating the seeds and encapsulating them in polyoxyethylene (Polyox), or by using hydrogel capsules (i.e., sodium / calcium-alginate), in case the plant species are recalcitrant. Capsules can be 1) placed directly for sowing (no need for acclimatisation), 2) placed on nutrient media until formation of plantlets, 3) stored in a laboratory fridge at 4 °C for short-term storage, or 4) stored in LN2 for long-term storage (Qahtan et al. 2019). For further details on production, techniques, protocols, and examples, see Faisal and Alatar (2019).

Animals

Invertebrate cultures

Different techniques are employed for culturing invertebrates (i.e., to maintain in captivity for research or teaching purposes), and they depend on the organism group. Culture and maintenance of micrometazoans, coelenterates, scyphozoans, bryozoans, polychaetes, and small crustaceans can be found in Smith and Chanley (1975). Culture of sea hares, nematodes and fruit flies can be found in Smith et al. (2011). Methods and protocols for culturing eutardigrades can be found in Degma (2018) and Roszkowska et al. (2021). Further methods for aquatic and terrestrial invertebrates can be found in Thorp (2015), and recommendations in Rogers and Thorp (2015).

Animal cell cultures

In vitro cell cultures are an important tool for *ex situ* conservation due to their potential to maintain a renewable source of high-quality genetic material (e.g., genomic DNA) for in principle unlimited periods without affecting animal welfare (Houck et al. 2017; Ryder and Onuma 2018; Cardoso et al. 2020; Sano et al. 2022). Cell cultures have been used for genomic and evolutionary studies, conservation (e.g., genetic rescue), toxicology, host-pathogen interactions, and medical research (Ezaz et al. 2009; Bui-Marinos et al. 2022), as well as for cloning, generation of induced pluripotent stem cells (Ben-Nun et al. 2011; Borges and Pereira 2019; Iqbal et al. 2021) and even for food production (FDA 2018; Goswami et al. 2022).

RECOMMENDATION
Approval from institutional animal care and use committees must be granted before starting a project. Moreover, animal care guidelines must always be followed to avoid animal pain. Nerve responses should also be monitored, and biopsy

samples should be collected within one hour of euthanasia.

Tissues to be used for the establishment of cell cultures are mostly collected post-mortem, usually during necropsy or surgical procedures (Houck et al. 2017; Bui-Marinos et al. 2022; Logan et al. unpublished), or when carcasses are discovered in the field (Calle and Ramírez 2022; Mattos et al. 2022). It is crucial that tissues are not necrotic, decomposed and do not bear signs of disease. Non-invasive and minimally invasive techniques can also be used, especially for threatened or rare animals (Ezaz et al. 2009; Moghanjoghi et al. 2018; Cardoso et al. 2020b), which depending on the size, must be anaesthetised (Praxedes et al. 2018). The collector should be a specialist in animal capture and should be familiar with laboratory-based tissue cultures, in case sample processing occurs under field conditions (Ryder and Onuma 2018). Feathers, placenta tissues at birth, gonads, skin, eye, trachea, lung, and kidney tissues are the main sources for the establishment of fibroblast cultures (Houck et al. 2017; Ryder and Onuma 2018). Blood samples can also be used, but collection may cause bleeding and stress to animals. Furthermore, it is difficult to obtain enough blood from small animals (Ezaz et al. 2009).

RECOMMENDATION
Sterility should always be maintained during sample collection, processing, and establishment of cultures. Fungi and bacteria can inhibit cell growth and even kill the cells. All laboratory steps must take place inside a laminar hood using sterilised equipment.

Before sample collection, the tissue surface should be wiped down with 70% alcohol. Collected tissue should be washed with sterile balanced salt solutions (e.g., PBS) and antibiotics, dried on cellulose filter paper, and minced into small pieces (0.5–1 mm^2) using a sterile scalpel blade or fine scissors (Ezaz et al. 2009; Wong et al. 2012; Siengdee et al. 2018; Bui-Marinos et al. 2022). If skin biopsies are taken, subcutaneous tissue (e.g., connecting tissue) and the front and back of the skin can be separated (Siengdee et al. 2018).

Ideally, the sample should be cultured immediately, but if this is not possible, it can be placed in a vial containing growth medium or PBS and antibiotics, stored within 24 h post-mortem at 4 °C up to five days (Wong et al. 2012; Houck et al. 2017; Praxedes et al. 2018; Strand et al. 2020; Sano et al. 2022). During transportation, samples should be carried in a chilled carrier (e.g., styro-foam container) and must not be in direct contact with ice packs to avoid sample freezing (Siengdee et al. 2018). Note that storage time for non-mammalian vertebrate tissues may be shorter than for mammals (Wong et al. 2012). If long-term storage of primary tissues is necessary, the vial should contain a cryoprotectant in concentrations between 5% to 15% (usually 10%) covering the whole sample, and it should be first placed at -80 °C in a CoolCell, Mr Frosty, or any other freezing container (cooled at 1 °C/min) for 24 h before being transferred to LN2 (Freshney 2010; Wong et al. 2012; Strand et al. 2021; Sano et al. 2022). Initiating cultures from tissues that were previously frozen and thawed will take longer and cultures will, in general, grow slower (Ryder and Onuma 2018), as freezing may affect cell adhesion and growth rate (Sano et al. 2022). Wong et al. (2012) and Sano et al. (2022) developed sample protocols for freezing tissue biopsy samples from wild animals prior to the initiation of cell culture.

RECOMMENDATION
The tissue culture medium is a crucial factor determining cell growth. Hence the choice of medium should be considered in advance. Moreover, the partial replacement of medium may be beneficial, as endogenous growth factors will accumulate in cultures.

Two methods exist to establish cell cultures: the explant and the enzymatic digestion method. In the former, the explant tissue adheres to the vessel surface, so that fibroblasts can migrate out of the tissue, whereas in the latter, the tissue is disaggregated by using an enzyme such as collagenase or trypsin for 30 min or longer, depending on the type of material (Cardoso et al. 2020b). Note that explants might lead to a more short-lived cell culture, especially relevant for small samples (Wong et al. 2012). Coating mixes (e.g., FNC coating mix) can also be added to improve cell adhesion (Sano et al. 2022).

RECOMMENDATION
Cell outgrowth should be regularly monitored using a phase contrast or inverted microscope by recording observations and taking pictures. The following parameters should be assessed: number of attached and subconfluent explants, day when explants attach, day when explants reach subconfluence, total time required to complete subconfluence, and total culture duration (Praxedes et al. 2021). If microbial contamination is observed, the culture should be immediately discarded.

Tissue should be transferred to a cell culture vessel and incubated at 37 °C for mammals, 38–40 °C for birds (Cardoso et al. 2020b), 26–32 °C for reptiles (Fukuda et al. 2012; Moghanjoghi et al. 2018; Kulak et al. 2020; Logan et al. unpublished), 20–30 °C for amphibians (Strand et al. 2021), and at a broad temperature range (20–32 °C) for fish (Lakra et al. 2011; Goswami et al. 2022), being 28 °C the optimum for marine fishes (Yashwanth et al. 2020). Cells usually start growing within 4–5 days and cell migration and proliferation may take 1–2 months or longer depending on the tissue, the growth conditions (Bui-Marinos et al. 2022) and the group of organisms (amphibian and reptile growth rates are lower than in mammals and birds) (Camilla Di Nizo, pers. comm.). Subculturing is performed by detaching the cells from the vessels by trypsinization or mechanical means when the cells reach 70–90% confluence, or when cell growth is excessively dense (Phelan 1998; Ezaz et al. 2009; Ryder and Onuma 2018; Praxedes et al. 2021; Bui-Marinos et al. 2022; Logan et al. unpublished). The obtained cell suspension can then be split into fresh cultures (Phelan 1998). Bui-Marinos et al. (2022) provide guidance in determining when cells from a tissue explant culture should be subcultured.

Sample collection (including post-mortem biopsies), processing and cell culture protocols for different vertebrates can be found in Wong et al. (2012) and Houck et al. (2017). Primary culture methods (i.e., first culturing event) and cryopreservation methods can be found in Freshney (2010) and Ryder and Onuma (2018). Guidance on good cell culture practice and on fundamental techniques in cell culture can be found in ECACC (2018).

Note: primary cell lines, which are not immortalised (i.e., secondary cell lines) still retain the features of the original tissue from which they were isolated and have not yet undergone any genotypic or phenotypic variation. Therefore, they can be a proxy for studying the individual itself (Kroglund et al. 2022).

Note that these cell cultures are not technically immortalised, implying that after many passages they will reach a state where they undergo ageing and stop propagation (Galli and Lazzari 2021). Ideally, established finite cell cultures with no signs of senescence and in early passages (3–12 events) should be used for cryopreservation (Logan et al. unpublished; Bui-Marinos et al. 2022), because there is a lower risk of karyotype abnormalities or epigenetic alterations (Galli and Lazzari 2021). Cells to be cryopreserved should be harvested and washed by adding a salt solution (e.g., HBSS, PBS) and a dissociating solution (e.g., Gibco TrypLE, trypsin) (Strand et al. 2021). Prior to freezing, cells can be counted in a Neubauer chamber, if necessary (Cardoso et al. 2020b).

Note that most of the following cell culturing protocols developed for different taxonomic groups also include their corresponding cryopreservation protocols, and hence are not included in the cryopreservation chapter of this handbook.

In vitro cell cultures can be assessed by performing the same procedures applied after somatic cell cryopreservation recovery (see retrieval and viability chapter). Briefly, cell morphology and membrane integrity, mitochondrial dehydrogenase activity (MMT), population doubling time (PDT), and chromosomal quantification are analysed for culture characterisation (Praxedes et al. 2018).

Note: the identification of the cell type, the characterisation of cells during long culturing periods and the assessment of cryopreservation methods needs to be considered when establishing a cell bank (Praxedes et al. 2021). See Kaplan and Hukku (1998), Geraghty et al. (2014), Yu et al. (2015) and Almeida et al. (2016) for guidelines and standards regarding characterisation and quality control of cell lines. **The Cellosaurus** is an online resource for authentication of cell lines using STR profiles.

Marine invertebrates. Fifty years ago, the development of cell cultures in marine invertebrates started, especially from sponges, cnidarians, molluscs, crustaceans, echinoderms, urochordates and cephalochordates. Until recently, permanent establishment of cell lines was not possible, (Rinkevich 2005; Cai and Zhang 2014; Conkling et al. 2019), due to cellular quiescence after 24–72 h of cell isolation (Rinkevich 2011; Potts et al. 2020). Currently, some successful cell lines are being established for sponges and cnidarians (Kawamura et al. 2021; Urban-Gedamke et al. 2021).

Sponges are used whole as tissue source, with the difference (to the procedure described above) that the sponge should be sieved to remove large aggregates of cells, right after cutting the specimen into little pieces (Conkling et al. 2019). Apical branch tips from corals, hemolymph from crustaceans and molluscs, and guts from echinoderms are mainly used as a source tissue (Cai and Zhang 2014). Although it is also possible to use other types of tissue, embryonic tissue seems to be the most promising for all phyla (except Porifera) to initiate cultures. Note that the developmental stage of the embryo will affect the effectiveness of cell culture approaches (Cai and Zhang 2014). The Cnidarian Cell Culture Consortium is currently establishing cell line protocols for the taxon (Roger et al. 2022). Cai and Zhang (2014) reviewed several protocols published until 2012 for all the phyla mentioned above, whereas Zahiri and Zahiri (2016), Rosner et al. (2021) and Ballarin et al. (2022) reviewed different studies regarding stem cells in marine invertebrates. Further details can be found in Mitsuhashi (1989).

Refer to Schippers (2013), Conkling et al. (2019) and Urban-Gedamke et al. (2021) for protocols in sponges, Barnay-Verdier et al. (2013), Ventura et al. (2018), Fricano et al. (2020) and Kawamura et al. (2021) for cnidarians; to Jayesh et al. (2012), Söderhäll (2013), and Potts et al. (2020) for crustaceans; to van der Merwe et al. (2010), Yoshino et al. (2013), Maselli et al. (2018), and Suzuki et al. (2021) for molluscs; and to Wang et al. (2020), and Rodríguez-Hernández (2020) for echinoderms.

Arthropods. Cell cultures have mainly been established to study biological control of insect pests and disease vectors (Mitsuhashi 1989). Hence, several cell lines, especially for lepidopteran species, are available (Lynn 2007a). Hemolymph from larvae or embryos can be used to initiate cultures (Guo and Weng 2020). First, eggs should be immersed in 70% alcohol and rinsed with sterile distilled water. The eggs are opened to release the embryo, which is separated from the yolk and macerated. Incubation occurs at 27 °C (Johnson et al. 2010). Specific tissues from larvae can also be used to establish cultures following Lynn (2007b). **Protocols** for ticks and other arthropod disease vectors (midges, sand flies) have been developed by Bell-Sakyi et al. (2019). For hymenopterans, see Hunter (2010), Goblirsch et al. (2013), Guo and Weng (2020), and Watanabe et al. (2021).

Refer to Lynn (2002) for maintenance of insect cell cultures. Further culture conditions can be found in Drugmand et al. (2011) and Maramorosch and McIntosh (2017).

Other invertebrates. Protocols have been developed for the freshwater polyp *Hydra* (Li et al. 1963) and for the nematode *Caenorhabditis* (Strange et al. 2007; Zhang et al. 2011, Zhang and Kuhn 2005–2018). Refer to Mitsuhashi (1989) for protocols in land slugs and nematodes, and to Fuller-Espie et al. (2015) and Riedl et al. (2022) for earthworms. Some first attempts have also been made for tardigrades (Haldrup et al. 2015). Bayne (1998) provides recommendations and considerations for insects and worms.

Fish. Several fish cell lines have been reported (Nagpure et al. 2013). Generally, skin explants from the caudal fin are the main source for establishing cell cultures (Chenais et al. 2014), although gills, eye, heart, kidney, muscle, and liver tissues have also been used (Nagpure et al. 2013; Goswami et al. 2022). Note that the skin needs to be wiped thoroughly with sterile gauze before sampling to remove the build-up mucous, which can lead eventually to culture contamination (Wong et al. 2012). The choice of temperature for culture incubation will depend on the species, but usually it is 20–23 °C for cold-water fish, and 26–30 °C for warm-water fish (Bols et al. 2011).

Standard necropsy procedures and culture maintenance protocols can be found in NWFHS (2004) and Bols et al. (2011). Lakra et al. (2011) provide an overview regarding development, characterisation and storage of fish cell lines, as well as a list of cell lines that have been developed for different freshwater fishes.

Amphibians. Skin biopsies can be taken from the hind legs, or the posterior end of both dorsal and ventral sides, but other kinds of tissues (e.g., tongue, eye) can be used (Zimkus et al. 2018). Strand et al. (2022) evaluated and validated a protocol for different types of tissues, since most cell line protocols were developed more than 20 years ago. Bui-Marinos et al. (2022) optimised a step-by-step explant protocol for skin cell cultures that can be adapted to many frog species by adjusting the culture medium and solutions. A protocol for the establishment of cell cultures from the great crested newt has been developed by Strand et al. (2021). Recommendations for initiating, establishing, and maintaining frog cell lines can be found in Bui-Marinos et al. (2022).

Reptiles. Primary fibroblast cultures can be established from tail (5–10 mm) and toe clips (3 mm) or toe webbing, but also from blood, heart, spleen, kidney, and internal connective tissue (Ezaz et al. 2009; Logan et al. unpublished). Reptile cells can be maintained at a range of different temperatures, possibly because a reptile's temperature adjusts to the environment (Fukuda et al. 2012; Moghanjoghi et al. 2018). A non-invasive protocol can be found in Ezaz et al. (2009) and can be applied to a wide range of reptile species. Logan et al. (unpublished) developed a universal protocol for terrestrial reptiles, whereas Moore et al. (1997) and Fukuda et al. (2012) developed protocols for marine turtles.

Birds. Blood feathers are the preferred source for initiating cell cultures, followed by blood (Kroglund et al. 2022). Blood feathers (i.e., developing feathers) from the wings or tail should be pulled out at the base and the pulp removed from the calami to establish fibroblast cultures (Houck et al. 2017). Feathers in early developmental stages are preferred because they contain larger amounts of pulp tissue than mature feathers (Cardoso et al. 2020). Refer to Sasaki et al. (1968), Romanov et al. (2009), Cardoso et al. (2020), and Kroglund et al. (2022) for protocols.

Mammals. Fibroblasts from skin and foetal fibroblasts are the most common cell types used in mammals (Galli and Lazzari 2021), although other tissue sources such as muscle, bone marrow, intestines, adipose tissue, and oral mucosa have been used (Praxedes et al. 2018; Calle and Ramírez 2022). Skin biopsies, mainly from the groin area or the abdomen, or ear notches (1–2 cm^2 for medium-size (e.g., agouti), 2–3 cm^2 for large animals) can be obtained, for instance, during veterinary exams or radio-collaring, or from hunters (Houck et al. 2017; Sano et al. 2022). Note that hair and fur are a source of contamination, so they should

be removed before sampling by shaving and cleaning the skin with a gauze soaked in 70% alcohol. If shaving is not possible, the biopsy area should be washed with soap and water, dried, and subsequently cleaned with 70% alcohol for 15–20 s. Harsh disinfectants can harm cells and should be avoided (Wong et al. 2012).

Protocols, recommendations, and challenges to establish fibroblast cultures can be found in Ma et al. (2011), Siengdee et al. (2018), Praxedes et al. (2021), and Sano et al. (2022). Phelan (1998) developed protocols for human cell cultures that can be used for other mammals. Species-specific protocols have also been developed, among others, for agouti (Costa et al. 2020), rabbit (Gavin-Oagne et al. 2020), peccary (Borges et al. 2020), Iberian lynx (Leon-Quinto et al. 2009; 2011), puma (de Oliveira et al. 2021a), jaguar (de Oliveira et al. 2021b), buffalo (Selokar et al. 2018) and bats (Crameri et al. 2009; Yohe et al. 2019).

Environmental samples

Soil and Sediment

Soil samples and sediment cores can be stored in paper bags and sealed containers, respectively. Samples should preferably be kept in cold (4 °C), dark, and ideally, oxygen-free conditions to maintain the microbial community, constrain bacterial growth and decrease metabolic processes (Vandeputte et al. 2017; Capo et al. 2021; Duan and Bau 2021; Brasell et al. 2022). Depending on the type of sediment, samples should be kept for up to two (e.g., for lacustrine material) or six months to prevent loss of DNA (Brasell et al. 2022). High temperature-drying (60 °C) of soil for 12 h is also possible but microbial diversity and reliable sequencing precision may be affected (Duan and Bau 2021). Note that storage as well as sediment type (e.g., clay, organic) will impact the abundance of some organisms in the samples (Tennant et al. 2022).

Many environmental specimen banks (ESBs) or other biobanks store soil samples at -80 °C rather than in LN2 for long-term storage (Clasen et al. 2020; Duan and Bau 2021; Schroeder et al. 2021). However, it is recommended to snap freeze samples – including those preserved in e.g., DNA/RNA buffers or DESS – in LN2 and store them in the LN2 gas phase (Rüdel and Winsgärtner 2008; Pavlovska et al. 2021).

Soil samples should be placed in cryovials and frozen right after collection (Rüdel and Winsgärtner 2008; Duan and Bau 2021). The cooling chain should not be interrupted during transport (Rüdel and Winsgärtner 2008). Samples can undergo a cryomilling procedure, meaning they should be ground and homogenised under cryogenic conditions (-130 °C) using, e.g., an oscillating mill or a planetary ball mill in a laminar flow hood (Rüdel et al. 2008). It is critical that the samples do not warm up during the process. Hence, mills and vessels have to be cooled to -150 °C using LN2, following LN2 safety regulations (ISBER 2019). If the sampled material has a large grain size (<1 cm), stainless-steel mortar and pestle, either pre-cooled or immersed in LN2, should be used beforehand. This procedure can also be applied to filters (Rüdel and Winsgärtner 2008; Rain-Franco et al. 2021).

RECOMMENDATION
Freeze-thaw cycles increase the proportion of DNA from facultative anaerobic and metabolically versatile bacteria, inducing a bias in the signal from bacterial communities found in sediment core samples. Hence, it is recommended to purify samples on site or store them instead at 4 °C for up to ten weeks (Capo et al. 2021; Tennant et al. 2022).

RECOMMENDATION
Ideally, soil and sediment samples should not be stored in nucleotide preservatives such as RNA later because samples can be damaged due to the possible interaction with humic acid.

Sediment cores can be cryopreserved at -80 °C, but it has to be noted that subsampling of cores should be done before freezing to avoid compromising their stratigraphic integrity (Brasell et al. 2022). Sediment subsamples should be taken immediately after opening the core and stored at -20 °C or -80 °C to reduce sample degradation due to fungal and bacterial growth, and cross-contamination (Massana et al. 2015; Capo et al. 2021; Duan and Bau 2021; Tennant et al. 2022). The CAS-freezing technology has also been used to preserve seafloor sediment samples (Braun et al. 2016; Trembath-Reichert et al. 2017).

Water and air samples

Water samples and filters used for water and airborne samples can be stored at -20 °C until further processing (Egeter et al. 2018; Johnson et al. 2019; Ovaskainen et al. 2020; Rhymes et al. 2021). Recommendations for preservation of freshwater samples are provided in Baricevic et al. (2022).

Palaeontological samples and human/ archaeological remains

Bone and tooth samples can be stored in a dark and cool place without temperature fluctuations (Mitchell and Brickley 2017; Fulton and Shapiro 2019); whereas hair, skin and other preserved soft tissue can be stored frozen, in separate containers (Funck et al. 2020). Guidance on storage of specific human remains (e.g., collagen, dry tissue, wet samples) is provided in SMA (Society for Museum Archaeology 2020).

Non-charred archaeobotanical remains should either be stored in glass tubes containing a mixture of glycerine, alcohol, and water in equal quantities or freeze-dried (Charles et al. 2009). Alcohol should not be added if the samples contain any resin (Ward 1998). To avoid fungal growth, waterlogged seeds should be thoroughly dried, before storing them in air-tight glass vials. Fragile and small seeds can be kept in gelatine capsules, which are also placed in glass vials. Charred material can be wrapped in cotton tissue and stored in glass or plastic containers (Charles et al. 2009). All samples should be stored in a cool and dry environment, attempting to maintain the conditions under which the samples were found.

Further details regarding the storage of bio-archaeological remains can be found in Monk et al. (2007) and Campbell et al. (2011).

CHAPTER 5
Cryopreservation

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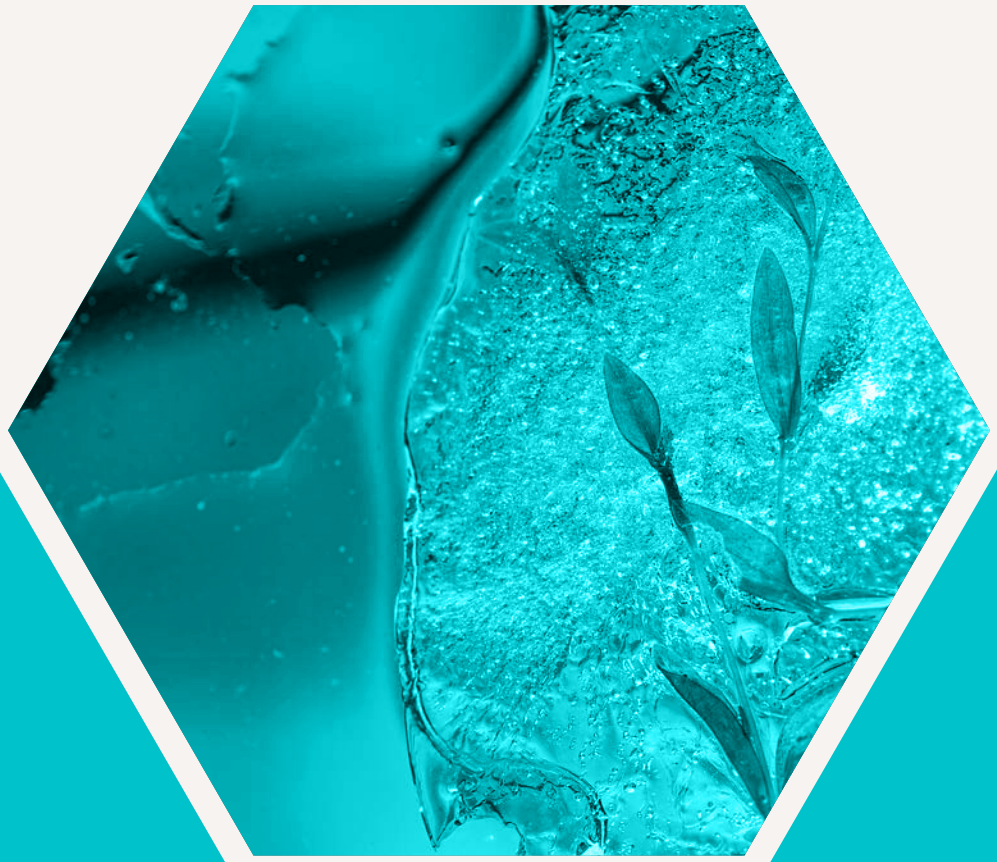

Introduction

Cryopreservation, in the narrow sense of the word, is the use of ultra-low freezing temperatures to conserve living cells, tissues, etc. in a state of suspended animation, ensuring not only cell viability but also the conservation of high-quality DNA and other biomolecules (Elsen et al. 2007; Rawson et al. 2011; Daly et al. 2018; Zomerdijk et al. 2020; Castillo et al. 2021; Gallichotte et al. 2021; Narida et al. 2022). In a wider sense, cryopreservation and other terms including the root "cryo-" simply denote ultra-low frozen storage, usually involving the use of liquid nitrogen, but without necessarily encompassing sample viability. In this chapter, we follow the narrower definition and focus on viable samples. Cryopreservation can also be applied to environmental samples, to viably preserve the microbial community composition along with intra- and extracellular DNA (Duan and Bau 2021; Baricevic et al. 2022).

Most biological material has to undergo several preparation steps prior to cryopreservation, especially if it does not endure immediate submersion into LN2 (Elsen et al. 2007). The most challenging factor when developing cryopreservation protocols is finding the optimal cooling rate for each taxon or strain (Tedeschi & de Paoli 2011; Smith and Ryan 2012; Homolka 2014; Singh and Baghela 2017; Ribeiro et al. 2022). Exceedingly slow cooling rates will cause cell damage due to high concentration of electrolytes, while overly fast rates will favour damaging intracellular ice formation (Paredes et al. 2018; Castillo et al. 2021; Funnekotter et al. 2021; Narida et al. 2022). Furthermore, other factors must be considered for successful cryopreservation, such as sample volume, cell type and its biophysical and biochemical properties, medium type, cryoprotectant type and concentration, exposure time and cryoprotectant removal, and embryo developmental stage (Paredes 2016; Paredes et al. 2017; Campbell et al. 2020; Andrae et al. 2021; Raju et al. 2021; Moreira et al. 2022). In animals, the plasma membrane composition (especially the aquaporin channels), and its permeability features should be considered in protocol development, since the plasma membrane is involved in the transport of water and solutes (Delgado-Bermúdez et al. 2022; Ribeiro et al. 2022). A detailed description of the freezing process can be found in Kilbride and Meneghel (2021).

Note that the most frequently used cryoprotectants are dimethyl sulfoxide (DMSO) and glycerol, which are toxic as their concentration increases (Campbell et al. 2020; Raju et al. 2021). Storage of frozen samples in LN2 is implemented either in vapour-phase (-190 °C to -165 °C, even up to -130 °C) (Webb et al. 2018; Funnekotter et al. 2021) or liquid-phase (-196 °C).

Cryopreservation methods have not been widely standardised, and a universal protocol that would hold for all species or genera does not exist (Brito et al. 2017; Hagedorn et al. 2019). Thus, protocols must be carefully adapted to the specificity of each taxon and cell type to maximise the potential for cryobanking (Lermen et al. 2009; Martínez-Páramo et al. 2016; Cardoso et al. 2020a). Wolkers and Oldenhof (2021) provide the most updated protocols to cryopreserve various types of cells as well as basic knowledge about the principles of cryobiology.

RECOMMENDATION

Fungi and bacteria may be present in LN2 tanks, especially in the ice layer underneath the tank lid, which are likely originating from the stored material. Therefore, it is recommended to minimise the ice formation and to use hermetically sealed tubes to avoid potential contamination of the samples (Bajerski et al. 2020). General recommendations to reduce the risk of microbial contamination, as well as an assessment spreadsheet for quality management can be found in Bajerski et al. (2021).

Ideally, personnel involved in cryopreservation procedures should be qualified or should have obtained the required training to carry out this work. When cryopreserving biological material, several cryovials should be prepared as back-ups and for viability assessments (CCAP 2021). Polypropylene or polycarbonate cryotubes with ring seals and internal or external threads, or with sealing lips should be used for tight close, thus avoiding contamination, tube damage (Homolka 2014), and chemical alteration of the sample or preservation fluid.

RECOMMENDATION
Specific information regarding the cryopreservation procedure should always be recorded for each cryovial: unique ID on vial and original voucher ID, date and person who placed in LN2, location in LN2 tank, taxon name, pre-treatment conditions, growth medium, cryopreservation method and used cryoprotectants, name and number of plant propagules per vial, and viability controls (Funnekotter et al. 2021).

Microorganisms

The use of vigorous and actively growing cultures under stress-free conditions is the key factor for a successful cryopreservation, as these will show high levels of viability during the revival procedure (Day and Harding 2008; Bégaud et al. 2012). Moreover, cultures selected for cryopreservation should be in a dense state and in a mid- to late-stationary phase, meaning they should have been growing for at least one month (CCAP 2021). They should also be free of microbial contaminants. Cryopreservation is highly recommended for dictyostelids, amoebae, former zygomycetes and oomycetes, phytopathogenic fungi and yeasts (Nakasone et al. 2004). Furthermore, most culturable cyanobacteria, soil microalgae and marine diatoms can undergo cryopreservation (Day 2007; Day and Harding 2008; Day et al. 2017).

To avoid shipping expenses of frozen material, strains need to be first grown on agar or in a liquid medium before distribution at room temperature (Nakasone et al. 2004). Two protocols can be performed but are dependent on the species sensitivity: the slow-controlled-freezing protocol using a controlled-rate cooler or a container with metal core (e.g., Mr. Frosty, CoolCell), and the fast-freezing protocol. The former is more commonly used, often applying a freezing rate of 1 °C/min (Homolka 2013; EmbaRC 2012) to prevent intracellular ice formations (Ryan and Ellison 2003; Day et al. 2005).

Protists

Long-term preservation of protists is mainly achieved by subculturing and only cyst forms are stored frozen (Altermatt et al. 2015). Yet, ciliate cysts have been successfully cryopreserved, using vitrification methods (Müller et al. 2008). In principle, cryopreservation using DMSO as cryoprotectant presumably works for all protist species, including the parasitic forms (Tedeschi & de Paoli 2011). However, low survival after thawing may still occur. Therefore, the following recommendations should be followed to increase survival rates (Altermatt et al. 2015):

- Thawing and survival rates should be experimentally determined for each taxon/strain and cryopreservation method (e.g., cryoprotectant concentration between 5–12.5%) before it becomes a routine method.
- Eight cryovials should be prepared per culture to increase survival rate.
- Controlled cooling-down should precede LN2 storage.
- Frozen samples should never be kept outside LN2 storage for any longer than 1 min.

- Frozen cultures should be regularly reinitialised to assess genetic variation.

Parasitic blood protozoa, however, can be frozen without cryoprotectants and without an accurate cooling rate control (De Paoli 2005). Protocols for parasitic forms can be found in Diamond (1995), Miyake et al. (2004), Murilla et al. (2016), and Jaskiewicz et al. (2020).

In addition to the mentioned generic protocol for many protists (Altermatt et al. 2015), Liu et al. (2019) established a cryopreservation protocol for marine ciliates with a broader application to other marine protozoa. Folgueira et al. (2018) also developed a protocol specifically for marine ciliate endoparasites. Free-living amoebae protocols can be found in Seo et al. (1992). Further cryopreservation and freeze-drying protocols for specific protozoa species can be found in Lee and Soldo (1992).

Regarding microalgae, methanol and DMSO are the preferred cryoprotectants (Day et al. 2005). Yet, many microalgae with complex morphology (e.g., euglenoids), filamentous algae, and large-cell-sized algae are recalcitrant to current cryopreservation procedures (Harding et al. 2004; Day and Harding 2008; Day et al. 2010; Tessarolli et al. 2017; Kapoore et al. 2019; Paredes et al. 2021a). The presence of rigid cell walls and vacuoles usually hampers the penetration of the cryoprotectant into the cells and complicates the freezing process (Day 2005; Paredes et al. 2021a). Pre-culture conditions, cryopreservation protocols and further recommendations can be found in Mori et al. (2002), Day (2007), Day and Harding (2008), Gäbler-Schwarz et al. (2013), Day et al. (2017), Paredes et al. (2021a), and Arguelles et al. (2020). Furthermore, Day and Brand (2005) provided several detailed cryopreservation and thawing protocols that have been applied to a broad range of microalgae. Harding et al. (2008) also developed an encapsulation/dehydration method for microalgae. Cryopreservation protocols for marine microalgae have been established by Rhodes et al. (2006) and by the ASSEMBLE+ project (Paredes et al. 2020a). Step-by-step cryopreservation protocols for *Euglena* and related genera were developed by Harding et al. (2010) and Shah et al. (2022). Customised protocols for chlorophytes are provided in Fernandes et al. (2019) and for marine dinoflagellates in Kihika et al. (2022). A list of successful cryopreservation approaches in various microalgae species can be found in Nugroho et al. (2016) and in Paredes et al. (2021a).

Phenotypic characterisation of microalgae using a miniaturised growth measurement should also be performed before and after cryopreservation to determine the success of the procedure (Day et al. 2005).

Fungi and fungus-like organisms

Cryopreservation is considered the best preservation procedure for fungi and fungus-like forms (e.g., Mycetozoa, Oomycota). Yet, changes both in hyphae morphology and viability during freezing and thawing have been recorded when storing cryopreserved strains at merely -80 °C (Ryan et al. 2000). Both sporulating and nonsporulating cultures, as well as those that cannot be lyophilised or have delicate spores, can be kept at ultra-low temperatures (Nakasone et al. 2004; Agarwal and Sharma 2006).

Spores, mycelia cultures, and air-dried conidia can be cryopreserved, using cryoprotectants such as glycerol, trehalose, or DMSO to reduce injuries during the cryoprocess, although the latter is often toxic for sensitive organisms (Hubálek 2003; Ryan and Smith 2004; Homolka 2013; Singh and Baghela 2017).

Some cultures can be frozen directly after attaining suitable growth (Abd-Elsalam et al. 2010). Otherwise, mycelia agar should be cut from the margin of the colony with a sterile cork-borer or a sterilised plastic straw. The excised disks (ca. 8–10 disks with 4 mm diameter each) should be placed in cryovials containing 10% glycerol. This method applies to mycelia that grow deep into the agar or fungi that do not sporulate. Five tubes should be prepared in this way from each isolate (Vasas et al. 1998; Nakasone et al. 2004; Overy et al. 2019). Polypropylene vials must be placed directly into the vapour phase of LN2 (Nakasone et al. 2004).

Mycelia growing in liquid culture should macerated and fragmented in a miniblender prior to

pipetting. An equal part of 20% glycerol should be added to the vial (Nakasone et al. 2004). Mycelia growing on the agar´s surface can be gently scratched with a pipette and placed in a cryovial. Spore suspensions should ideally be placed in polypropylene straw ampoules together with 10% glycerol. Both mycelia and spores should be precooled at 7–4 °C, then prefrozen at -40 °C, at a rate of 1 °C/min, followed by a rate of 10 °C/min until reaching -90 °C. Ampoules and vials should be immediately submerged into LN2 (Juarros et al. 1993; Nakasone et al. 2004; Agarwal and Sharma 2006).

Most cultures are stored in mechanical freezers at -80 °C. Fungi and fungus-like forms growing on different organic substrata (e.g., cereal grains, agar strips, filter paper), and that do not sporulate excessively, should be dried before freezing (Nakasone et al. 2004; Abd-Elsalam et al. 2010). Deep-freezing at -80 °C is not recommended for ectomycorrhizal fungi (Heinonen-Tanski and Holopainen 1991). Cryopreservation protocols for oomycete cultures can be found in Kitamoto et al. (2002), Uzuhashi et al. (2020), and Eszterbauer et al. (2020).

Preservation on porous beads. This was originally developed for storing bacteria in the LN2 vapour-phase (Homolka 2014). Beads (e.g., ceramic or glass beads) work as carriers and have been used for nonspore-forming fungi (Lakshman et al. 2018), sporulating fungi and yeasts (Homolka 2013). Beads should be taken with sterile forceps out of their vial and placed in a Petri dish containing the culture for up to seven days, or until the beads have been covered with mycelia. Beads should be returned to the original vial after removal of the contained preservative solution. Preservation temperatures can be either -80 °C or -150 °C (Homolka 2013). Commercial Microbank beads should never be immersed in LN2 (Lakshman et al. 2018). Cryopreservation of air-dried or suspended conidia can also be carried out using porous or polystyrene beads as carriers (Chandler 1994).

Preservation on perlite. Perlite is a unique aluminosilicate volcanic mineral that can retain water and release it when needed (Homolka 2013). Perlite has been suggested as a favourable substitute for serial transference used with agar cultures in long-preservation of fungi (Simões 2013). This method has been successfully applied to yeasts, Ascomycota, Zygomycota, and some Basidiomycota strains that cannot survive other types of preservation (Homolka 2013). Perlite is used as a carrier of mycelia and can be added directly to the cryovials. For further details, refer to Homolka et al. (2007). The protocol is described in Homolka (2013).

RECOMMENDATION

The maintenance of Basidiomycota is challenging because most of them do not form asexual spores, and their mycelia are sensitive to environmental conditions. Always include a prefreezing step, as direct immersion of strains into LN2 or -80 °C will be detrimental. A detailed review about cryopreservation of Basidiomycota can be checked in Linde et al. (2018). Additionally, a comparison of preservation methods can be found in Palacio et al. (2014). Preservation protocols can be found in Eichlerová and Homolka (2014).

Recalcitrant species such as the water mould *Saprolegnia* spp. and unculturable fungi such as microcylic rusts can be cryopreserved by vitrification and immobilisation or encapsulation (Ryan 2001; Eszterbauer et al. 2020). The vitrification technique has not been broadly tested on fungi, so further investigation is needed (Homolka 2014; Singh and Baghela 2017). Immobilisation is a technique involving alginate encapsulation of mycelium/spores prior to preservation (Ryan and Smith 2004; Ryan et al. 2019). Simões (2013) has used this method for preservation of recalcitrant fungal strains and several filamentous fungal species maintained in 10% glycerol. The combination of encapsulation and vitrification may be appropriate for mutualistic associations such as orchidaceous mycorrhizal fungi (Kermode et al. 2012), endophytes and lichens (Ryan and Smith 2004), as well as for obligate pathogens, which must be cryopreserved together with their hosts (Homolka 2014; Singh and Baghela 2017; Strittmatter

et al. 2020). Plants used as hosts should go under a quarantine period prior to inoculation with infected plant material (Ryan and Ellison 2003).

Ryan and Ellison (2003) developed a protocol for cryopreservation of microcylic rusts. However, their pathogenicity/infection ability was not successful after retrieval, and spores did not survive after direct plunge freezing. The authors recommended the use of stem or petiole tissue, as leaf material may have moisture issues after cryopreservation.

Some institutions have specialised in specific types of fungi and have developed their own preservation protocols. For instance, the **West Virginia University** has developed methods for spore extraction, hyphal harvesting, culturing, voucher preservation and storage of vesicular arbuscular mycorrhizal fungi. The methods include conservation of *in vivo* pot cultures in sterile soils or other supporting materials, *in vitro* cultures with genetically modified root-organ culture of host plants, and *in vitro* autotrophic systems in artificial media with axenic plants (Drazhnikova and Andrianova 2020).

Further cryopreservation protocols for fungi can be found in **Westerdijk fungal biodiversity Institute**. Protocols for yeasts and filamentous fungi can be found in Bond (2007) and Ryan and Smith (2007), respectively.

Information regarding macrofungi can be found in Linde et al. (2018) and Ilyas and Soeka (2019). Mata and Pérez-Merlo (2003) and Mata and Savoie (2013) established protocols for preservation of macrofungal mycelia on cereal grains without using cryoprotectants. Furthermore, Sato et al. (2020) assessed a novel cryopreservation protocol that uses vermiculite grains – a soil additive – to improve the viability of ectomycorrhizal basidiomycetes (e.g., *Amanita* spp.) that are difficult to cryopreserve.

Lichens

Lichens can be cryopreserved either in LN2 or at -80 °C (Pence et al. 2020). Cryopreservation can be performed for each individual symbiont using cryoprotectants as well as cooling devices such as MrFrosty or a controlled-rate freezer. Alternatively, dried thallus fragments can be cryopreserved without cryoprotectants and slowly inserted into LN2 tanks, thanks to the lichens´ ability to survive at extreme temperatures in a dormant state (Honegger 2003).

Benthic algae

Cryopreservation has mainly been employed for long-term preservation of microalgae and cyanobacteria. Only recently, efforts have started to develop conventional cryopreservation and vitrification protocols for macroalgae that are important for aquaculture and as model organisms (e.g., *Saccharina latissima*, *Ulva* spp.) (Heesch et al. 2012; Paredes et al. 2018; Day 2018, Kapoore et al. 2019; Visch et al. 2019, Yang et al. 2021). Protocols have focused on cryopreservation of gametophytes, vegetative thalli, and spores (Bhattarai et al. 2007; Zhang et al. 2007; Zhang et al. 2008; Lalrinsanga et al. 2009; Heesch et al. 2012; Lee and Nam 2016; Barrento et al. 2016; Visch et al. 2019). It is crucial to initiate culture preparations two weeks prior to the procedure. Approximately, 2 mm of thallus should be removed and placed into an Erlenmeyer flask containing culture medium, which should be changed once a week. In this way, axenic and vigorous cultures can be achieved (Day 2018). Protocols and comments, as well as a list of successfully cryopreserved macroalgae, can be found both in Day (2018) and Paredes et al. (2021a). Cryopreser-

vation protocols for red seaweed and for sugar kelp can be found in Visch et al. (2019), Field and Campbell (2020) and Perrineau et al. (2020). Furthermore, a complete review regarding germplasm cryopreservation plus a summary of cryopreservation protocols applied to macroalgae is provided in Yang et al. (2021). The ASSEMBLE+ project has also developed cryopreservation approaches for more than 200 algal species (Paredes 2019). Note that encapsulation methods are not recommended for seaweeds, nor for marine microalgae, as high sodium levels in marine media will depolymerise the encapsulating alginate matrix (Paredes et al. 2018).

Plants

Plant conservation efforts have focused mainly on seed banking of crops and of rare species (Pence et al. 2002), especially in the light of current loss of landraces, cultivars, and varieties (Hodkinson et al. 2007; Panis et al. 2020). Furthermore, plant biotechnology, including *in vitro* technologies and cryopreservation, plays a crucial role in the conservation of plant genetic resources, especially for varieties and species that do not set seeds or produce recalcitrant seeds (including many tree species and tropical species). However, the application of these techniques is challenging due to the great variety of plants (including intraspecific level) and their responses (FAO 2014).

RECOMMENDATION
Although it is possible to cryopreserve samples coming directly from the field, grown in vitro material is preferred, especially, when small amounts of material have been collected. Thus, material can be multiplicated in aseptic cultures, and cryoprotectants can be added during preculture stages, if necessary.

Cryopreservation of embryonic axes, shoot tips, axillary buds, somatic embryos, calluses, and suspension cultures

So far, cryopreservation is the only available method providing long-term conservation for plant genetic resources that can be propagated only vegetatively, and for recalcitrant and rare species (Engelmann 2009; Pence et al. 2020; Funnekoter et al. 2021). The physiology and ecological characteristics of each species must be considered to identify the most optimal cryopreservation technique (Sarasan et al. 2006; Sarasan 2010; Engelmann 2010; Pence et al. 2020). Various strategies have been developed to adapt cryopreservation protocols efficiently (Kim et al. 2012; Funnekotter et al. 2021):

Slow cooling procedures. Freezing temperatures are usually applied to cell suspensions, calluses, and dormant buds, as well as to cold-tolerant species. This process involves slow cooling to about -40 °C, followed by a rapid immersion in LN2. A protocol designed for plant cell suspensions can be found in Grout (2007). Jenderek et al. (2010) gives an overview on dormant bud cryopreservation.

Vitrification technique. This approach uses a highly concentrated solution of different cryoprotectants such as DMSO, glycerol, sucrose, sorbitol and/or ethylene glycol, which allows cells to dehydrate and to vitrify during freezing. Cryoprotectants can be used individually or in combination. The most commonly used plant cryoprotectant is PVS2, a combination of DMSO, glycerol, sucrose and ethylene glycol (**protocol**) (Panis and Lambardi 2005; Funnekotter et al. 2021). Other cryoprotectants are PVS3 with 50% sucrose and 50% glycerol (Nishizawa et al. 2008; Senula and Nagel 2021) or PVS1, PVS4 or Vitrification Solution L (VSL) (Zamecnik et al. 2021). Instead of using pure DMSO (Schäfer-Menuhr et al. 1996), potentially

toxic for the cells, propylene glycol can be used as a replacement. Vitrification methods can be highly reproducible and can be applied to different types of tissue and to a broad range of species (Panis and Lambardi 2005). It has been noted that plant propagules may age during LN2 storage, potentially due to the inherently short lifespans of certain species and due to the fact that cell architecture may change after vitrification processes (Ballesteros et al. 2021). Further research on this topic is needed.

Droplet vitrification method. Tissues (e.g., apices) are treated with droplets of a vitrification solution on aluminium foil strips that are rapidly frozen in LN2. This method is the most widely used as it can be applied to a wide range of species (and genotypes) including woody and herbaceous species (Wang et al. 2021). A step-by-step guide to developing new protocols can be found in Panis et al. (2011).

Cryo-plate techniques. Calcium alginate capsules containing tissue (e.g., shoot tips) are secured on aluminium cryo-plates. The vitrification cryo-plate (V Cryo-plate) method is a combination of encapsulation-vitrification and droplet vitrification. The dehydration cryo-plate (D Cryo-plate) combines the encapsulation with the dehydration method, and usually shoot tips are air-dried. Handling becomes easier, as the cryo-plates are manipulated instead of the plant material. Both methods are promising for herbaceous and woody plant preservation after modifications of the original protocols (Yamamoto, et al. 2011; Matsumoto 2017).

Cryo-mesh technique. It is comparable to the cryo-plate techniques, but the main difference is that a stainless-steel mesh strip is used instead of a cryo-plate (Wang et al. 2021).

Desiccation technique. The plant material, mainly zygotic embryos and embryonic axes, is dehydrated using a stream of compressed air or under the laminar flow of a lab bench on silica gel and, then immersed in LN2 (Sakai et al. 2000).

Encapsulation–dehydration technique. This method is comparable to the synthetic seed technology, as the plant material (e.g., shoot tips) is also encapsulated in alginate beads. Beads are then dehydrated, either by air-drying or using silica gel, and then immersed in LN2. Some of the advantages of this technique include easier tissue manipulation, protection during dehydration, and no need for cryoprotectants (Niino and Sakai 1992).

Encapsulation-vitrification technique. Tissue samples are encapsulated in alginate beads and then submitted to freezing by vitrification. A protocol for shoot tips and meristems can be found in Benson et al. (2007).

The use of other tissue samples such as small leaf square-bearing adventitious buds (SLS-BABs), stem disc-bearing adventitious buds (SD-BABs), rhizome buds and microtubers can simplify cryopreservation protocols, as the most time- and labour-consuming step –the shoot tip excision– can be excluded (Wang et al. 2021). However, dormancy, viability and recovery of the tissue are factors to consider in decision making.

RECOMMENDATION
Ideally, genetic and epigenetic stability should be assessed in the plantlets produced after cryopreservation, because *in vitro* culture, cryoprotectants, and some vitrification-encapsulation steps might induce genetic and epigenetic variations.

Several protocols for cryopreservation can be found in:

- Specific journals such as Acta Horticulturae, Journal of Plant Physiology, Plant Cell, Tissue, and Organ Culture (PCTOC), Frontiers in Plant Science, Methods in Molecular Biology, Plant Science, Propagation of Ornamental Plants, Physiologia Plantarum, and Journal of Horticultural Science and Biotechnology.
- Bettoni et al. (2021) provide information regarding facilities, technical skills, and plant material conditions to implement plant shoot tip cryopreservation.
- **The Laboratory of Tropical Crop Improvement at KU Leuven** defined cryopreservation protocols for 26 crop species such as banana, taro, and common chicory.

- Wang et al. (2021) provided a comparison of new technologies for *in vitro* based cryopreservation, as well as a reference list for several cryopreservation protocols.
- Cruz-Cruz et al. (2013) provided an extensive reference list for protocols in different taxa.
- Thammasiri et al. (2019) compiled cryopreservation protocols for crops.
- Reed (2008) provided step-by-step instructions for developing new cryopreservation protocols based on well-established and successful protocols. It includes protocols for the cryopreservation of both orthodox and recalcitrant seeds, pollen, dormant buds, bryophytes, ferns, de-differentiated cell cultures, embryogenic cultures, embryonic axes, crops, herbaceous plants, and trees.
- Engelmann and Takagi (2000) included papers from a workshop, describing different protocols for cryopreservation of tropical plants.
- Normah et al. (2019) suggested strategies for cryopreservation of shoot tips of recalcitrant and tropical species.
- Popova et al. (2015) reviewed different cryopreservation protocols for diverse plant species. They also provided strategies for developing protocols *de novo*. See also Popova et al. (2016, 2019, 2020).
- Engelmann (2011) provided an overview on embryo cryopreservation and on in vitro derived propagules (Wang et al. 2021).
- A vast list of species that have been cryopreserved, using seeds, cell suspensions, calluses, embryonic axes, somatic embryos, and shoot-tips can be found in Gonzalez-Arnao et al. (2014).
- A recent compilation of cryopreservation protocols for different organisms and tissues, including plants, can be found in Wolkers and Oldenhof (2021).
- Roque-Borda et al. (2021) reviewed some cryopreservation methods applied to agronomic plant germplasm.
- A special issue on "**plant cryopreservation**" in the journal Plants (2021:10) includes new protocols, physical and chemical aspects of freezing and drying, cold adaptation and thawing.

The following table offers a list of protocols developed for different plant species:

Table 4. List of protocols developed for different plant species.

Type of plant	Method	Reference
Medicinal plants	Root cryopreservation Shoot tip cryopreservation	Yang et al. (2019) Senula et al. (2018)
Musaceae	Cryopreservation of apical meristems, embryogenic cell suspensions and zygotic embryos	Panis et al. (2005) Panis (2009)
Potato	Cryopreservation of shoot tips	Vollmer et al. (2017) Köpnick et al. (2018)
Grapevine	Cryopreservation of shoot tips	Bettoni et al. (2019a) Bettoni et al. (2019b)
Apple	Cryopreservation of shoot tips* Cryopreservation of dormant buds	Bettoni et al. (2020)* Bettoni et al. (2019c) Bettoni et al. (2018) Hofer (2015)
Tobacco	Suspension cell culture cryopreservation	RIKEN BioResource Research Center, Kyoto, Japan (2019)

* **Photos and videos** are available to observe the cryopreservation process.

Cryopreservation of bryophytes and ferns

Bryophyte spores can be dried to low RHs (<50%) and stored dry at low (sub-zero) temperatures. Survival to LN2 exposure after drying to 15%RH has recently been reported, opening the doors to bryophyte spore banking (Tiloca et al. 2022). Bryophyte spores often contain chloroplasts and storage lipids, potentially affecting sample longevity (Ballesteros et al. 2020), thus monitoring is recommended. Bryophytes also produce gemmae, undifferentiated vegetative propagules, that can be collected and placed in small paper envelopes. Envelopes should be placed in a box containing silica gel for ca. 3 h (time may vary depending on the species) for drying. Envelopes containing dried gemmae should be placed in cryovials and directly immersed in LN2 (North et al. 2021).

Fern spores should be dried in environments between 15–75% RH and stored at either -20 °C (Ballesteros et al. 2019), -80 °C or at -196 °C to maintain their long-term (> 10 years) viability (Nebot et al. 2021). However, storage conditions must be carefully defined for each species as some species may age faster at -20 °C when compared to higher (e.g., 5 °C) or lower (-80 °C, -196 °C) temperatures potentially due to lipid crystallisation issues (Ballesteros et al. 2019). For short- (1–3 years) and medium- (up to 10 years) term storage, refrigeration (3–7 °C) can be used after drying at a RH between 15% and 25% (Ballesteros et al. 2019; North et al. 2021). Storage at room temperature or wet storage is not recommended (Ballesteros and Pence 2018). Spores may deteriorate and die, particularly chlorophyllous/green spores, during LN2 storage due to their inherently short lifespan (Ballesteros and Pence 2017; Ballesteros et al. 2019). Longevity will vary depending both on the temperature and the type of spore, with species having chlorophyllous/green spores ageing faster than non-chlorophyllous/non-green spores (Ballesteros and Pence 2018; Ballesteros et al. 2019).

In addition to the spores, bryophyte and fern gametophytes and sporophytes can be cryopreserved for long-term conservation purposes (Ballesteros and Pence 2018). These tissues are generally cryopreserved following the encapsulation dehydration method. This procedure comprises three steps: 1) the encapsulation of tissue in spheres of alginate gel, 2) cryoprotection using a combination of osmotic dehydration with concentrated sucrose and drying, and 3) rapid immersion in LN2. For ferns, whole gametophytes can be used, but if too large, they should be cut into small pieces prior to cryopreservation. The use of abscisic acid during tissue culture likely improves survival during LN2 storage (Ballesteros and Pence 2018). Furthermore, actively sporophyte-growing meristematic tissue, such as shoot tips or green globular bodies survives better than mature non-growing tissues (Ibars and Estrelles 2012). This method has been mainly applied to leptosporangiate ferns, while further research is needed for eusporangiate and lycophyte genera (Ballesteros and Pence 2018). Refer to Mikula et al. (2011) and Nebot et al. (2021) for gametophyte and spore protocols respectively. Further information and protocols can be found in Pence (2008). For bryophytes, techniques similar to those employed for ferns can be used. Rowntree et al. (2005; 2007; 2009; 2011) developed several LN2 cryopreservation protocols for UK bryophyte species. Cold-acclimated bryophyte specimens have also been successfully cryopreserved without the use of cryoprotectants; the protocol can be found in Segreto et al. (2010).

Cryopreservation of pollen

In general, mature orthodox pollen does not need additional treatments before cryopreservation (Ganeshan et al. 2008; Funnekotter et al. 2021). Protocols for pollen cryopreservation both for desiccation-tolerant pollen and for a desiccation-sensitive pollen species (e.g., corn) can be found in Nebot et al. (2021). Protocols for different commercially relevant species can be found in Ganeshan et al. (2008) and for tropical plant species in Rajasekharan et al. (2013). Further cryopreservation protocols are described in Popova et al. (2015).

Recalcitrant pollen should be quickly and partially desiccated to moisture levels at which no freezable water exists but above levels

where desiccation injury is apparent (Towill and Walters 2000; Anushma et al. 2018). Current desiccation techniques can be found in Nebot et al. (2021) and Impe et al. (2022).

Cryopreservation of seeds

Orthodox seeds. In general, orthodox seeds have a low moisture content and can be stored in LN2 without cryoprotectants. Seeds should be dried at 25–32% RH and then, they can be placed into cryovials and immersed in LN2. If seeds have a large size, they can be placed in laminated foil packets (Funnekotter et al. 2021).

Orthodox seeds with short-life spans. Some orthodox seeds have a very short-life span (i.e., *Populus deltoides* or *Salix* spp.) and tend to deteriorate and die within a few years (Pammenter and Berjak 1999). Although low temperature storage under low moisture content is possible, cryopreservation may increase longevity of these seeds. However, it has to be noted that drying requirements for LN2 storage will be different from those for conventional storage. Seeds are mostly dried at 32% RH and 18 °C or 5 °C before cryostorage (Ballesteros et al. 2021). The Cincinnati Zoo and Botanical Garden suggests a list of publications and videos on how to evaluate the best storage for short-lived seeds. Also note that seeds may continue ageing and degrading while maintained in LN2 (Walters et al. 2004; Ballesteros and Pence 2017; Davies et al. 2018). Walters et al. (2004) concluded that cryopreservation does not maintain cells in an infinite longevity state, as it has been established that molecules continue moving at temperatures below -130 °C, allowing ageing to advance (Ballesteros and Walters 2019; Ballesteros et al. 2020). To mitigate this, mature seeds must be freshly harvested, should have a high initial quality, and require careful handling (Ballesteros and Pence 2017). Ballesteros et al. (2021) provided cryopreservation methods for orthodox and intermediate seeds, as did Pritchard (2007) and Pritchard and Nadarajan (2008).

Intermediate seeds. To avoid drying damage, intermediate seeds are often dried to higher moisture contents than those for conventional storage. Drying of intermediate seeds can be done at 20-25 °C and 50-75% RH before cryopreservation storage (Ballesteros et al. 2021; Funnekotter et al. 2021).

Recalcitrant seeds. This type of seeds usually has a large size. Hence, the embryonic axes or the embryo can be excised and cryopreserved (Funnekotter et al. 2021). This procedure should take place right after collection in a laminar flow hood under sterile conditions. Embryonic axes need to be processed within two hours after removal. Embryos should be flash-dried using silica gel or saturated salt solutions in a drier for ca. 2-4 hours of rapid drying (Berjak et al. 1993), then to be placed into cryovials and stored in the vapour phase of LN2 (Ballesteros et al. 2021). It is crucial that embryos are at the right developmental stage for a successful cryopreservation (Theilade and Petri 2003). Refer to Ballesteros et al. (2021) for detailed guidance on the excision of embryonic axes method. Recalcitrant seeds can also be germinated *in vitro* and shoot apical meristems from the seedlings can be used for cryopreservation as an alternative when embryo excision is not possible (FAO 2014). Further protocols for working with recalcitrant seeds can be found in Walters et al. (2008) or Ballesteros et al. (2021). Cryopreservation is thus recommended for long-term storage.

Excision of embryos from orthodox and intermediate seeds is also possible. However, prior seed desiccation should be avoided (Funnekotter et al. 2021).

Animals

Animal cryopreservation has mainly focused on germplasm (e.g., sperm, spermatogonia, epididymal semen, oocytes, and primordial germ cells), embryos/larvae and somatic cells

(Lermen et al. 2009; Martínez-Páramo et al. 2016). Cryobanked material can subsequently be used among others for breeding, aquaculture, genetic improvement, conservation, and restoration purposes, as well as for research to answer questions in genomics, transcriptomics, and proteomics (Lermen et al. 2009).

Several protocols have been developed for sperm cryopreservation, which is considered the best-established technique for different taxa. Note, however, that sperm obtained from the epididymis cannot be treated as the sperm obtained using typical methods, as it requires special handling before cryopreservation (Bertol 2016). The cryopreservation workflow includes andrological examination, semen collection, dilution, centrifugation, resuspension of the pellet with the freezing medium, packaging, freezing and post-thaw sperm evaluation (Yánez-Ortiz et al. 2021). Determining sperm concentration prior to cryopreservation, using micro-spectrophotometric methods, is crucial. Primary and freezing extenders are typically used to dilute sperm (e.g., Beltsville or Lake fluid) and avoid motility activation (Tiersch 2001; Martínez-Páramo et al. 2016). The storage packaging (e.g., pellets, straws, Cryolock, Fibreplug) is also important, as the material and shape will affect the cooling rate during the freezing and thawing procedure (Martínez-Páramo et al. 2016).

Cryopreservation and post-thaw recovery of oocytes and embryos from non-mammalian species, such as birds, amphibians, or fishes, have so far not been successful due to their larger size, low surface-to-volume ratio, fatty yolk, high chilling sensitivity, susceptibility to intracellular ice formation, and low cell permeability (Saragusty and Arav 2010; Long 2013; Robles et al. 2017; Comizzoli 2017; Daly et al. 2018; Browne et al. 2019; Campos et al. 2021; Clulow et al. 2022). However, oocytes in their early stages seem to be more responsive to cryopreservation than mature and later stages (Rawson et al. 2011). The use of ovarian follicles can be an alternative to oocytes and embryos, as they are smaller and have a less complex membrane system (Martínez-Páramo et al. 2016). Moreover, most oocytes and embryos do not tolerate high concentrations of cryoprotectants (Woelders et al. 2018; Hagedorn et al. 2019). Yet, the use of new devices, such as closed and open vitrification systems (e.g., open pulled straws, Cryotop, Cryoloop, Cryotip, Fibreplug, Vitrisafe, Kitasato system) and quartz microcapillary may allow reduction of those concentrations by increasing cooling and warming rates (Daly et al. 2018; Gonzalez-Plaza et al. 2022; Narida et al. 2022; Nisa et al. 2022). The use of vitrification methods together with laser absorbers (e.g., India ink, gold nanoparticles) diluted in the cryoprotectant solution, and subsequent laser warming could be an alternative to oocyte and embryo/larvae cryopreservation (Khosla et al. 2017; Daly et al. 2018; Hagedorn et al. 2019).

Other methods that have emerged as an alternative to the use of oocytes and embryos involve the cryopreservation of primordial germ cells to produce viable gametes, and the transfer of spermatogonial stem cells into host larvae from the same or different species (Robles et al. 2017; Clulow et al. 2022). Note that these procedures are currently problematic to perform in birds because the efficiency of germline chimeras is low (Woelders 2009; Yan et al. 2014).

Viable somatic cells have also been cryobanked with—among others—the aim of preserving diploid genomes for somatic cell nuclear transfer (SCNT) (Stacey and Dowall 2007; Singina et al. 2014; Hagedorn et al. 2019). Somatic cells can be collected independently of the sex or age of the individual (Martínez-Páramo et al. 2016). Immediately after collection, the tissue should be either frozen in small pieces (Moritz and Labbe 2008) or kept in culture at appropriate conditions to produce viable cells prior to cryopreservation (Ezaz et al. 2008; Rawson et al. 2011, Martínez-Páramo et al. 2016). Detailed information regarding somatic cell culturing and cryopreservation can be found in the "Culture preservation and storage" chapter.

Cryopreservation protocols have been developed for different taxonomic groups and some of them are mentioned below.

Invertebrates

Marine invertebrates. Lack of knowledge about reproduction biology and physiology of many marine organisms, as well as the short-term availability of gametes are also big challenges for developing cryopreservation protocols (Paredes 2018; Hagedorn et al. 2019). Paredes et al. (2018, see also cited references) provide a summary of protocols applied to different marine invertebrate organisms (molluscs, echinoderms, cnidaria, sandworms, barnacles), whereas Paredes (2015) focused mainly on protocols for sea urchin and bivalve larvae. Moreover, the CRYOMAR protocol toolbox (Paredes 2020a) provides protocols for larvae and sperm of mussels and sea urchins, as well as sperm from sea cucumbers and oysters.

A review on cryopreservation protocols in crustaceans and other marine invertebrates is provided in Guo and Weng (2020, see also supplementary material) and Aquino et al. (2022). Sperm protocols are described in Morales-Ueno and Paniagua-Chávez (2020) for crustaceans, in Demoy-Schneider et al. (2020) for bivalves, and in Hagedorn et al. (2019) for corals. Further research on the cryopreservation of gametes and larvae of echinoids, ascidians and polychaetes is currently being carried out by Paredes (2020b) and Paredes et al. (2021b). Toh et al. (2022) provide a somatic cell cryopreservation protocol for corals.

Campos et al. (2021) reviewed oocyte cryopreservation challenges in aquatic invertebrates. Coral larvae, as well as zebra fish larvae, have been successfully cryopreserved using vitrification and laser warming (Khosla et al. 2017; Daly et al. 2018).

Helminths. Many helminth species can only be cryopreserved using vitrification methods. Eggs, larvae and microfilariae are frequently cryopreserved applying a protocol that includes the addition of ethylene glycol in two steps, followed by rapid cooling to -196 °C (James 2004). Cryopreservation protocols for parasitic worms can be found in James (1985, 1990, 2004) and Eckert (1988). Other protocols have been developed for specific genera such as *Heterorhabditis* (Nugent et al. 1996), *Schistosomula* (James 1982), *Dirofilaria* (Shirozu et al. 2020; Zinser et al. 2021), and *Pristionchus* plus other free-living nematodes (Matthias Herrmann, pers. comm.). Elsen et al. (2007) focused on tropical and some other plant-parasitic nematodes, whereas Beraldo and Pascotto (2014) focused on animal-parasitic nematodes.

Insects. Cryopreservation techniques have been applied to the house fly, ladybird beetles, spined soldier bugs, fireflies, fruit flies, silkworms, honeybees, and eventually to mosquito species known to act as vectors for human pathogens (Gallichotte et al. 2021; Viert et al. 2021). Note that a developmental stage that is permeable to water and cryoprotectants has to be used for cryopreservation. Hence, embryos and eggs with chorion, wax layers or vitelline membranes are not recommended (Gallichotte et al. 2021). Ethylene glycol is generally used as a cryoprotectant in insects, and often trehalose or polyethylene glycol is added to avoid damage during the vitrification process (Gallichotte et al. 2021).

A robust and easy to implement protocol for fruit fly embryos can be found in Zhan et al. (2021). A protocol for honeybee semen can be found in Auth and Hopkins (2021), whereas Whitman et al. (2019) described a freezing preservation (-80 °C) protocol useful for molecular studies of terrestrial arthropods.

Vertebrates

Fish. Many fish sperm cryopreservation protocols have been developed, mostly for marine species (Rawson et al. 2011; García et al. 2016; Martínez-Páramo et al. 2016). Sometimes it may be necessary to pool samples from different individuals to obtain the required volume (Martínez-Páramo et al. 2016). Generally, 10–15% DMSO is effective for fish, but glycerol, trehalose, and propylene glycol have proven more effective in some species (Browne et al. 2019).

Cryopreservation protocols for sperm and germ stem cells, as well as the current state of cryopreservation of oocyte and embryonic cells can be found in Betsy and Kumar (2020), Routray (2020), and Cabrita et al.

(2022). Additional protocols are provided in Kopeika et al. (2007) and Rawson et al. (2011). Dhanasekar et al. (2022) developed a protocol for cobia sperm, and Yang et al. (2022) for brown-marbled grouper sperm. Protocols specifically designed for shark and ray sperm can be found in García-Salinas et al. (2021). Refer to Šćekić et al. (2019; 2020) for cryopreservation protocols of eel gonadal tissue and ovarian stem cells, and to Tiersch (2001) for sperm cryopreservation of aquarium fishes. A summary of germ cell cryopreservation in different teleost species is provided in Robles et al. (2017). See references cited in Martínez-Páramo et al. (2016) for further protocols.

The AQUAEXCEL project developed a protocol booklet for different fish species (Labbé 2018), as well as a validation procedure for the isolation, cryopreservation and transplantation of trout and carp germ stem cells (AQUAEXCEL 2020). In addition, the **AQUAGAMETE** project included the development and standardisation of techniques for cryopreservation of fish sperm.

Amphibians. Spermatozoa from anurans (as from fishes) can be kept at 4 °C for days to weeks without losing viability (Browne et al. 2019). When cryopreserved, 5–10% DMSO or DMFA (dimethyl formamide) should be used as cryoprotectants, but sucrose and trehalose have also proven to be successful (Browne et al. 2019). Rawson et al. (2011), Poo and Hinkson (2019), and Burger et al. (2022) have developed sperm cryopreservation protocols that can be applied to different threatened anurans. Further details regarding cryopreservation of maternal-haploid and embryonic-diploid genomes can be found in Clulow et al. (2022). Strand et al. (2020) also reviewed the topic and produced a summary of protocols and references.

Reliable methods for urodeles have not been established yet (Hagedorn et al. 2019). However, McGinnity et al. (2022) have provided the first steps for the storage and cryopreservation of semen from the North American giant salamander and Guy et al. (2020) for newt species. No storage or recovery protocols exist for caecilians (Clulow et al. 2022a), but some reproduction technologies are under development (Browne et al. 2022). Cryopreservation protocols of somatic cells can be found in Bui-Marinos et al. (2022).

Reptiles. Successful protocols for cryopreserving reptile spermatozoa are scarce (Campbell et al. 2020; Strand et al. 2020), and so far, no reports of offspring production using cryopreserved sperm have been published, most likely due to sperm degradation (i.e., low motility recovery) following cryopreservation (Clulow and Clulow 2016).

A cryopreservation protocol for spermatozoa of squamate reptiles (snakes, lizards and amphisbaenians) has been optimised by adding caffeine to increase motility of sperm after thawing (Campbell et al. 2020; Sandfoss et al. 2022). Young et al. (2017) developed one of the first sperm cryopreservation protocols in lizards. Young et al. (2022a) also compared cryoprotectants and possible combinations to optimise sperm cryopreservation protocols in lizards. Hobbs et al. (2022) assessed different extenders and freezing rates to enhance a sperm cryopreservation protocol for the Australian skink. Young et al. (2021, 2022b) and Blank et al. (2022) developed different protocols for snakes. Furthermore, Clulow at al. (2022) provided a review regarding the latest cryopreservation studies both for reptiles and amphibians.

So far, the only cryopreservation protocol among crocodilians is for spermatozoa of the saltwater crocodile (Johnston et al. 2017). Lamar et al. (2021) published the first collection, characterisation, and storage protocol of tuatara semen. Sirinarumitr et al. (2010) determined the efficiency of certain extenders in olive ridley turtles and hawksbill turtles. Ravida et al. (2017) developed a protocol for desert tortoise sperm, which may also be applicable to other members of the Testudinidae family.

Birds. Sperm cryopreservation is more challenging in birds than in any other vertebrate, since the filiform shape of the spermatozoa makes them more vulnerable to injury from manipulation procedures (Gee et al. 2004; Çiftci and Aygün 2018; Cardoso et al. 2020a). Furthermore, avian sperm is very susceptible to the osmolarity changes that occur during the freez-

ing-thawing process (Cardoso et al. 2020a; Castillo et al. 2021). Some strategies, such as the addition of zinc oxide, lipids, or sialic acid have been included to maintain sperm viability after cryopreservation (Zhandi et al. 2019; Castillo et al. 2021). Further details can be found in Wishart (2007) and Woelders (2021).

Most protocols have focused on poultry semen cryopreservation (Iaffaldano et al. 2016; Thélie et al. (2019; Tkachev et al. 2020; Lin et al. 2022). However, sperm cryopreservation protocols have also been developed and optimised, e.g., for the common parakeet (Dogliero et al. 2017), the peregrine falcon (Cardoso et al. 2020a), and the common pheasant (Castillo et al. 2021).

Gonadal tissues have also been cryopreserved when semen collection is not possible and as a viable alternative to preserving the female genome, with the aim of obtaining poultry offspring, using the allotransplantation technique (Liptoi et al. 2020; Fujihara et al. 2022). Hu et al. (2022) have also developed a cryopreservation protocol for poultry embryonic gonads. Somatic feather follicle cell cultures can be cryopreserved following Cardoso et al. (2020b).

Mammals. No standard procedure exists for the cryopreservation procedure of domestic mammal gametes, except for bulls (Walters et al. 2009; Yánez-Ortiz et al. 2021; Layek et al. 2022). Still, these protocols have been adapted to cryopreserve sperm from wild animals, such as elephants and capuchin monkeys, with some success. Wild species usually require *de novo* methodologies, since the quality of sperm is lower and less uniform, compared to the sperm from lab and livestock animals (Durrant et al. 2019). Cryopreservation of oocytes and embryos –especially blastocysts– may be possible, but it is still limited due to the low availability of wild animals as gamete/gonad donors (FAO 2012; Bhat and Sofi, (2021). Ovarian tissue can be a good source of oocytes, as it can overcome oocyte cryodamage and super-ovulation issues (Bhat and Sofi 2021). Testicular tissue has been widely cryopreserved, and its perspectives and current advances are described in Silva et al. (2020). Naitana et al. (2015) reviewed the main advantages and disadvantages caused by cryopreservation of sperm, oocytes, and embryos from domestic animals.

Note that in mammals, cryopreservation protocols can vary among species and breeds, and inter-individual differences can also occur, probably due to age or inbreeding (Holt 2000; Toledano-Díaz et al. 2020). Yánez-Ortiz et al. (2021) provide a summary about sperm cryopreservation in farm animals, as well as a detailed explanation regarding structural and molecular sperm alterations caused by the procedure. Some sperm cryopreservation protocols for domestic animals can be found in Hiemstra (2003), Walters et al. (2009), Sieme and Oldenhof (2015) and Galarza (2019). Livestock cryopreservation protocols have also been developed under the support of different projects such as EuReCa (2010), Globaldiv (Ajmone-Marsan and Globaldiv consortium 2010), and **IMAGE**. The **Jackson Laboratory** provides a step-by-step cryopreservation protocol in mice. O´Brien et al. (2019) investigated the effect of two freezing protocols on sperm from several endangered wild species. A special issue on "**New Challenges in Cryopreservation**" in the journal Animals (2022:12) includes recent developments in the use of cryoprotectants, protocols and strategies for cryopreserving mainly mammal germplasm. A summary of cryopreservation methods for ovarian follicles from different species can be found in Campos et al. (2019).

Embryos to be cryopreserved should follow the standard methods approved by the International Embryo Technology Society (Stringfellow et al. 2010). Saragusty and Arav (2010) reviewed the progress in oocyte and embryo cryopreservation by slow freezing and vitrification and summarised the protocols applied in different mammalian species. Furthermore, Bhat and Sofi (2021; and references therein) reviewed the current state of ovarian tissue vitrification in several wild animal species, whereas Tharasanit and Thuwanut (2021) did so for domestic animals. Keros and Fuller (2015) developed a cryopreservation protocol for mammalian oocytes, whereas Fuller and Paynter (2007) developed one for mammalian embryos.

Somatic cells (e.g., from ear tissue) can also be cryopreserved for future use (Woelders et al. 2012). Workflows describing the set-up of somatic-cell banks are found in Groeneveld et al. (2008) and Sekolar et al. (2018). Protocols are usually more generic across mammalian species, and some are referred to in de Lira et al. (2021). A well-detailed protocol can be found in Siengdee et al. (2018).

A list of mammal cryopreservation methods by taxa from 1970 to 2016 can be found in Charlton et al. (2018). The following list mentions some cryopreservation protocols published from 2017 onwards:

Table 5. List of cryopreservation protocols for mammals.

Taxon	Method	Reference
Canids	Cryopreservation protocol for testicular tissue of grey wolf	Andrae et al. (2021)
	Cryopreservation of semen from red wolf	Franklin et al. (2018)
Felids	Cryopreservation of somatic cells of wild felids	Praxedes et al. (2018)
	Cryopreservation of semen from clouded leopards	Zainuddin et al. (2020)
	Cryopreservation of testicular cells from felids	Bashawat et al. (2020)
	Cryopreservation of semen from African lion	Luther et al. (2017)
	Cryopreservation of somatic cells from puma	Lira et al. (2021)
	Cryopreservation of Siberian tiger epididymal spermatozoa	Ibrahim et al. (2022)
	Cryopreservation of feline oviductal organoids	Thompson et al. (2023)
Mustelids	Cryopreservation of ferret sperm	Toledano-Díaz et al. (2021)
	Cryopreservation of European mink stem cells and oocytes	Calle and Ramírez (2022)
	Cryopreservation of testicular tissue of black-footed ferret	Lima et al. (2020)
Bears	Collection and cryopreservation of polar bear sperm	Wojtusik et al. (2021)
Sirenians	Cryopreservation of somatic tissues from the Antillean manatee	Nascimento et al. (2022)
Cetaceans	Cryopreservation of bottlenose dolphin sperm	Sánchez-Calabuig et al. (2017)
Ungulates	Vitrification methods for dromedary camel embryos	Skidmore et al. (2021)
	Cryopreservation of spermatogonial stem cells and testis tissue of buffalo	Devi and Goel (2022)
	Cryopreservation of European bison germplasm	Duszewska et al. (2022)
	Cryopreservation of Iberian Ibex sperm	Esteso et al. (2018)
	Cryopreservation of epididymal spermatozoa of the Cantabrian Chamois	Martínez-Pastor et al. (2019)
	Cryopreservation of giraffe epididymal spermatozoa	Hermes et al. (2022)
	Cryopreservation of Addra gazelle spermatozoa	Wojtusik et al. (2018)
	Cryopreservation of epididymal sperm from roe deer	Santiago-Moreno et al. (2021)
	Cryopreservation of llama sperm	Arraztoa et al. (2022)
	Cryopreservation of boar semen	Monteiro et al. (2022)
	Cryopreservation of collared peccary skin-derived fibroblasts	Borges et al. (2020)
	Cryopreservation of collared peccary testicular tissues	Maria da Silva et al. (2021)
	Cryopreservation of collared peccary ovarian tissue	Campos et al. (2019)
	Cryopreservation in rhinoceros	Hermes et al. (2018)
	Cryopreservation of fibroblasts from Sumatran rhinoceros	Jenuit et al. (2021)

Taxon	Method	Reference
Proboscidea	Semen cryopreservation of Asian elephants	Arnold et al. (2017)
Bats	Cryopreservation of phyllostomid bat sperm	Hermes et al. (2019)
Lagomorphs	Factors affecting rabbit sperm cryopreservation	Kubovicova et al. (2022)
	Embryo vitrification in rabbit model	García-Dominguez et al. (2019)
	Improving rabbit semen cryopreservation protocol	Di Iorio et al. (2020)
	Cryobanking of rabbit somatic cells	Gavin-Plagne et al. (2020)
Rodents	Sperm cryopreservation of Australian plain mouse	Ferres et al. (2018)
	Agouti somatic tissue cryopreservation	Costa et al. (2020)
	Cryopreservation of agouti cell lines	Praxedes et al. (2021)
Primates	Cryopreservation protocol for testicular tissue in *Macaca fascicularis*	Jung et al. (2020)
	Sperm cryopreservation in pig-tailed macaque	Zainuddin et al. (2019)
	Sperm cryopreservation in marmosets	Arakaki et al. (2019a)
	Cryopreservation of golden-headed lion tamarin sperm	Arakaki et al. (2019b)
	Cryopreservation of bonobo sperm	Gerits et al. (2022)
Marsupials	Review on sperm cryopreservation in koalas	Johnston and Holt (2019)

Environmental samples

Environmental samples are mainly used to monitor biodiversity and hazardous substances in the environment. Long-term storage should preserve chemical traits, integrity of extracellular and intracellular DNA, and ideally, also the viability of cells contained in environmental samples (Rüdel and Weingärtner 2008; Rain-Franco et al. 2021). However, microorganisms may die during the cryopreservation and thawing process due to physiological stress or physical damage during freezing itself (Rosinger et al. 2022). Currently, it is not possible to culture most microorganisms due to the lack of knowledge about their physiological requirements. Hence, challenges and complications may occur when thawing and restoring cryopreserved microbial communities (Rain-Franco et al. 2021). Prakash et al. (2020) advocate more research on the cryopreservation of intact environmental samples.

Rain-Franco et al. (2021) developed a cryopreservation procedure for aquatic microbial communities that can be applied to filters and water samples by using 10% (v/v) sterile-filtered DMSO. (See supplementary material S1 in Rain-Franco et al. 2021, for a detailed protocol). Note that filters should not become dry, as this will cause cell damage. For preservation, cryovials should be kept for 15 min at 4 °C before flash-freezing in LN2 and then stored at -80 °C for long-term storage. Filtered-water samples stored in falcon tubes can be first dipped in LN2 for 30 s and then stored at -18 °C (Thieme et al. 2016). Filters can also be flash frozen and stored at -80 °C without any treatment for molecular analyses (Massana et al. 2015).

RECOMMENDATION
Storing samples at -80 °C does not prevent the formation of ice crystals that can affect the viability of cells in the long term. Consider cryopreserving at -196 °C in LN2 to maintain communities in a vitrified state and avoid cell damage. This, in turn, will lead to preserve the starting microbial community and allow reproducibility in follow-up studies.

CHAPTER 6
Freeze-drying or Lyophilisation

Carolina Corrales, Jackie Mackenzie-Dodds, María Paz Martín,
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Introduction

Although cryopreservation is currently the method of choice for long-term preservation, freeze-drying procedures are also an alternative, as samples are maintained in an anhydrous state (Anzalone et al. 2018). Freeze-drying is widely used in the pharmaceutical and food industry and their procedures have been extrapolated to research (Saragusty and Loi 2019; Comizzoli et al. 2022). Basically, the water content of the sample is reduced, sublimated under low pressure and then residual water is removed by desorption (Adams et al. 2015; Comizzoli et al. 2022). External protectant agents such as trehalose, sucrose, anhydrin, or heat shock proteins are used before dehydration to shield the samples from oxidation and structural breaks during the procedure (Saragusty and Loi 2019; Saragusty et al. 2020). Still, some damage to the cell membrane may occur due to freezing or drying (Keskintepe and Eroglu 2015). Ideally, a tunable diode laser absorption spectroscopy should be available to measure water content and sublimation (heating) rate. However, this equipment is rather expensive, and water content is measured instead by trial and error (i.e., considering the effect of volume sample, temperature of the condenser, distance from the condenser, size of vials and pressure) (Saragusty and Loi 2019). Note that high rates of sublimation can lead to material being released out of the vials into the drying chamber, causing potential contamination (Matejtschuk et al. 2019), as well as alteration of cellular structures due to excessive vapour production (Chen et al. 2019). Freeze-dried samples can be stored for up to 5 years at 4 °C or at ambient temperature, preferably with a desiccant (e.g., in the presence of silica gel) to prevent rehydration (Straube and Juen 2013; Kaneko et al. 2014). Samples can also be stored at -80 °C (Pearson et al. 2006).

General reviews about the lyophilisation process of biological material can be found in Labconco (2010), Matejtschuk et al. (2019), Rockinger et al. (2021), and on the **SP industries** website.

Microorganisms and fungi

This procedure applies to sporulating fungi, such as in yeast, Ascomycota, and allied conidial fungi (Ryan and Smith 2004; Freitas et al. 2011) and it is the preferred method for storing cyanobacteria (Metting 1994). It has also proved to be a successful method for few microalgae (Arguelles et al. 2020) and apicomplexan parasites (De Paoli 2005). It is unsuitable, however, for most Basidiomycota and mycorrhizal cultures (Homolka 2014).

The standard protocol comprises controlled freezing of 7–10-day-old specimen cultures/spore suspensions in ampoules, and drying them under vacuum, until reaching a water content less than 5% (Ryan and Smith 2004; Homolka 2014; Ryan et al. 2019). Trehalose or serum may be added to the suspending media to reduce damage that might occur during the process (Bégaud et al. 2012; Homolka 2014). The success rate can vary within isolates of the same species, and it will depend on the culture quality and how well the organism grows or sporulates (Agarwal and Sharma 2006). Preserved ampoules are easy to handle, and they can be stored up to 50 years at room temperature or at 4 °C (Caleza et al. 2017). Other vials that can be used to freeze-dry microorganisms are rubber-stoppered vials and double-chambered vials (Adams et al. 2015). Moreover, samples do not need to be revitalised before shipping, avoiding damages during transportation (Ryan et al. 2019). A protocol used at the Micoteca da Universidade do Minho can be found in Si-

mões (2013). Protocols for yeast can be found in Bond (2007) and for filamentous fungi in Tan et al. (2007) and Ryan and Smith (2007). A general protocol for fungi can be found in Nakasone et al. (2004) and for microalgae in Arguelles et al. (2020).

Benthic algae and plants

Macroalgae (seaweeds) are dried mainly for the food and pharmaceutical sectors using oven-drying, microwave-drying and freeze-drying methods until moisture content is less than 10% (Ling et al. 2015; Badmus et al. 2019). Seaweed samples are cut into small pieces for an even drying. After drying, samples are usually ground into a fine powder and kept in air-tight containers at -20 °C or -80 °C (Ling et al. 2015).

Fresh plant material is usually freeze-dried for chemical compound analyses (Moura et al. 2015; Roslan et al. 2020; Park et al. 2021), but it is also suitable for DNA extractions (Hsia, et al. 2010; Anderson et al. 2018). Leaves are the most used material, which can be freeze-dried right after collection or dried with silica gel for 36 h in glassine bags (Anderson et al. 2018). Plant tissue can also be stored for 3–4 h at -80 °C before freeze-drying, but if thawing occurs during the initial first freeze-drying step, cells can break and the DNA will be degraded (Anderson et al. 2018). Note that several factors may affect the drying time such as the moisture content and the leaf fragment size. Drying can be considered concluded when dry weight values are constant over time (Badmus et al. 2019). Freeze-dried samples can be placed in sealed bags or ground in microtubes and stored at -20 °C (Salminen 2003; Anderson et al. 2018).

Animals

Freeze-drying methods have been used mainly in sperm and occasionally in somatic cells (e.g., white blood cells, platelets, fibroblasts), as until now dried cells have lost their viability and biological activity and cannot be resumed upon rehydration (Adams et al. 2015; Anzalone et al. 2018). Although viability assessments show that cell membranes are damaged, the DNA is often well preserved and can be used for cutting-edge techniques such as intracytoplasmic sperm injection (ICSI) and somatic nuclear transfer (Iuso et al. 2013; Keskintepe and Eroglu 2015; Saragusty and Loi 2019; Wakayama et al. 2022), meaning that nuclear viability does not correspond to cell viability (Loi et al. 2008).

Before freeze-drying, a freeze-drying solution (e.g., TE buffer) containing trehalose and a calcium chelator (e.g., EGTA, EDTA) should be used to improve sperm DNA integrity and inhibit endonucleases that can fragment DNA (Kaneko et al. 2014; Keskintepe and Eroglu 2015; Rockinger et al. 2021). In addition, dried sperm should not be stored longer than a month at room temperature because chromosomal integrity can be jeopardised (Keskintepe and Eroglu 2015). A general review regarding freeze-drying procedures in mammals can be found in Gil et al. (2014).

The most common drying technique is freeze-drying, but in theory more approaches that avoid freezing are available such as air-drying, convective drying, evaporative drying, heat-drying, spin-drying, vacuum-drying, and vitri-drying (Saragusty and Loi 2019). Microwave- and laser-assisted drying methods are currently being developed to create amorphous trehalose matrices, as alternatives for preservation of biological material (Cellemme

et al. 2013; Madison et al. 2020; Wang et al. 2021). Freeze-drying protocols have been optimised by Keskintepe and Eroglu (2015), Kaneko et al. (2014), and Anzalone et al. (2018), who also reported technical improvements of ICSI using dry spermatozoa. Saragusty and Loi (2019) suggest using epididymal sperm rather than ejaculated sperm, as the former has better DNA quality.

Revival or imbibition of the samples is a crucial step that remains understudied. The approach of "adding just water" to rehydrate should be reviewed in more detail, as it seems that it can break the cell membranes due to the sudden exposure to water. A more gradual and slow process (e.g., rehydration in humidity chambers) or the use of saline solutions should be considered, as well as the effect of the temperature during rehydration (Adams et al. 2015; Saragusty and Loi 2019).

Moreover, if preservation protocols develop further (e.g., membrane ion channel activation, use of electroporation), so that xeroprotectants can enter the cell (Saragusty and Loi 2019), long-term storage would be possible at room temperature, and hence, a new set of standard operating procedures must be developed (Comizzoli et al. 2022).

On the other hand, freeze-drying protocols have been used as an optimal DNA preservation method for shipping and storage of tissue samples, when cell preservation is not the aim (Straube and Juen 2013). Note that DNA stored at room temperature can still degrade if dried samples are exposed to humidity and oxygen (Colotte et al. 2011).

Environmental samples

Soil samples can be freeze-dried after collection to preserve high quality DNA/RNA of microbial communities during transportation, without the need of a cold chain. It is not recommended for long-term storage, because microbial diversity can diminish already after one week of storage (Weißbecker et al. 2017). Protocols can be found in Castaño et al. (2016), Weißbecker et al. (2017), Schulz et al. (2019) and Clasen et al. (2020).

Long-term storage in cold environments is the standard for sediment cores, but cores can eventually become dry, changing the core biochemistry, and hence, affecting DNA analyses (Enevold et al. 2019). Vacuum freeze-drying is an alternative for sediment core preservation, especially those containing pollen grains (Tirlea et al. 2015). Note that collected cores are still stored frozen at -80 °C until freeze-drying (Tirlea et al. 2015). Freeze-dried samples should be maintained in secondary sealed containers to prevent degradation due to humidity.

Freeze-drying, besides ethanol or RNAlater, is also used to preserve faecal samples prior to transportation. Frozen samples can also be thawed, freeze-dried, and stored at room temperature (Bensch et al. 2022).

CHAPTER 7
Retrieval from Preservation and Viability Assessments

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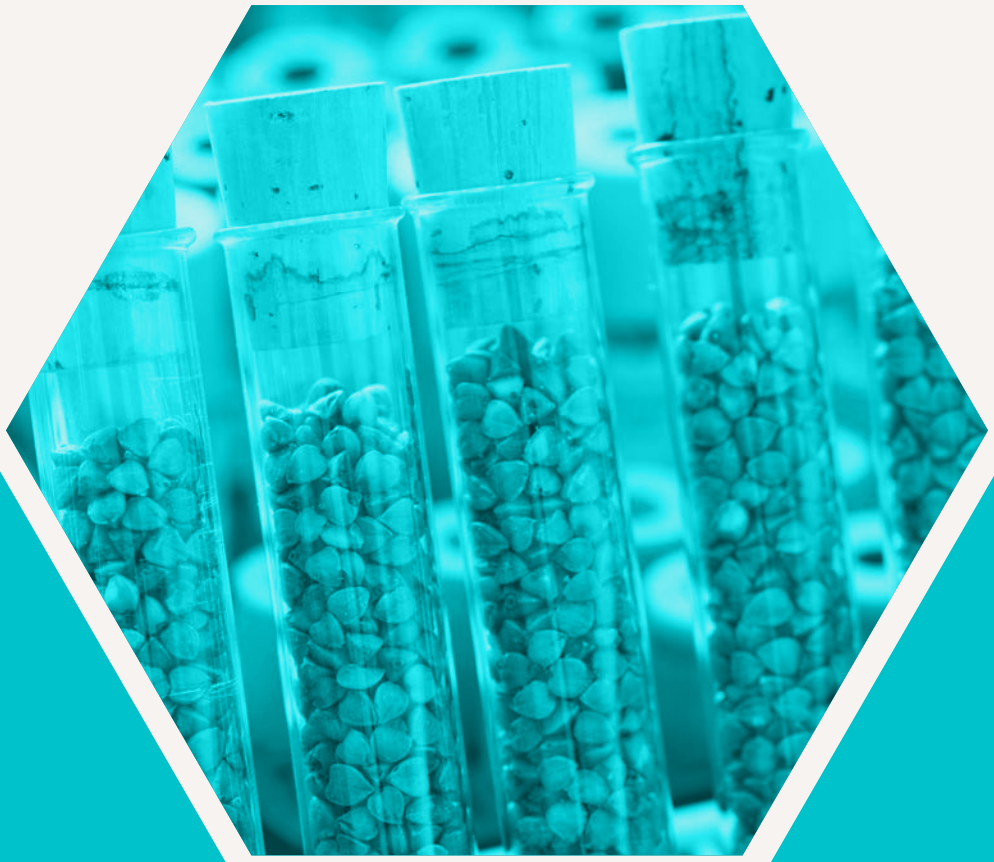

Introduction

Retrieval from any preservation method is an essential step to maintain the integrity of the samples post preservation (Ryan and Smith 2004). For instance, samples that are retrieved from cryopreservation storage can deteriorate during the warming procedure, causing physical damage to the plasma membrane due to ice crystallisation (Ryan and Smith 2004; Normah and Makeen 2008; Ballesteros et al. 2021). Note that this section focuses mainly on revival from cryopreservation procedures. The ISBER (2018) recommendations provide useful guidance on inserting or removing samples from LN2.

RECOMMENDATION
The use of a floating vial holder will reduce the risk of contamination that exists when vials are in contact with water in the water bath during thawing. The part of the tube containing the sample should be totally immersed for complete thawing and agitation should be avoided.

RECOMMENDATION
The outside of the cryovials should always be wiped with 70% ethanol, right after thawing and before opening the lid.

Protists and fungi

Recovery from filter paper, oiled or freeze-dried cultures is done by incubating a small amount of the culture onto an agar medium. The use of several subcultures may be necessary (Shivas et al. 2005; Agarwal and Sharma 2006; Singh et al. 2018). Freeze-dried yeasts and protists (e.g., free-living amoebae) need between 30–60 min to rehydrate –either in a broth or sterile distilled water at room temperature– before streaking on media (Shivas et al. 2005; Balczun and Scheid 2018). A guide to reviving lyophilized cultures is provided by the **Japan Collection of Microorganisms**. Further information regarding handling of cultures and rehydration of dried cultures can be found through the **German Collection of Microorganisms and Cell Cultures (DSMZ) website**. A protocol for reviving lyophilised algal cultures is available in Arguelles et al. (2020). Retrieval procedures from most preservation methods can be found in Singh (2017) and Singh et al. (2018).

Retrieval from cryopreservation must be performed carefully, and if toxic cryoprotectants have been used, they should be immediately removed by washing the samples (Ryan and Smith 2004). Usually, damage occurs when frozen microorganisms are exposed for extended periods of time to temperatures between -40 °C and -5 °C. Hence, thawing should be quick (Tedeschi and De Paoli 2011). Spores and mycelia should be placed in a water bath at 30–37 °C for ca. 3 min for thawing before opening ampoules/cryovials. Content should be streaked onto a growth medium (Agarwal and Sharma 2006; Rohadi et al. 2020; Sato et al. 2020). Depending on the species, cultures should be allowed to grow 1–14 days. Cryopreservation is considered successful when more than 50% of the thawed sample grows and morphological traits are retained (Vasas et al. 1998). If fungal samples were cryopreserved using porous beads, one single bead should be removed from the vial using sterilised forceps and streaked onto a solid growth medium using a loop. Vials should be placed into a cryoblock to avoid thawing of the whole sample (Homolka 2014; Chandler 1994).

RECOMMENDATION
If resources allow, the success of cryopreservation protocols can be assessed at different time intervals (e.g., once every two weeks, monthly, yearly).

Viability can be assessed by observing mycelial growth on a Petri dish with the naked eye and measuring the colony diameter (Homolka 2014; Sato et al. 2020). Alternatively, propidium iodide (PI) tests can be used to assess viability (Bohorquez et al. 2021), and germination tests can be executed with different concentrations of spores (e.g., Fries 1978, 1984; Martín and Gràcia 2000).

Teliospores are considered viable if a basidium, sterigma and basidiospores are produced. In turn, the basidiospores should produce a germ tube after incubation in a humidity chamber (Ryan and Ellison 2003). Conidia are considered viable when the length of the germ tube exceeds the width of the conidium. At least 300 conidia should be mounted in cotton blue and counted to assess germination success (Chandler 1994).

Pathogenicity can be assessed by inoculating the germinated fungal teliospores onto a host plant (Ryan and Ellison 2003). Further methods to assess strain stability following cryopreservation can be found in Smith and Ryan (2012).

It is recommended to revive cultures every 2–3 years to test their vitality (Vasas et al. 1998) as well as inspecting morphological aspects, such as mycelium colour, texture, zonation, growing margin, and colour of the reverse side (Da Lio et al. 2018).

Protozoa can be thawed at 37–42 °C for 30–120 s, depending on the taxon (Müller et al. 2008; Folgueria et al. 2018). A detailed protocol can be found in the **experimental microbial ecology repository of protocols**. Alternatively, protozoan samples can be placed in an ice bath until completely thawed (Santos-Pereira 2019). Protozoan viability can be tested using Trypan blue stain, and cells can be counted using a Neubauer chamber (Balczun and Scheid 2018). The CellTiter-Glo Luminescent Cell Viability Assay (Promega) is also an option for determining the number of viable protozoa in a culture (Botwright et al. 2020). Otherwise, cells can be live-counted using a Sedgewick-Rafter chamber (Müller et al. 2008; Folgueria et al. 2018).

Microalgae should be quickly placed in a water bath at 37–40 °C, and the samples can be gently agitated to help thawing. Samples should be transferred to culture media and kept in darkness overnight. Subsequently, they can be incubated under normal growth conditions (Day 2007; Day et al. 2016; Paredes et al. 2021). Additionally, Gouhier et al. (2020) developed a thawing protocol for cryopreserved marine microalgae.

Microalgal viability can be assessed using the fluorescein diacetate (FDA) staining method or by combining it with chloromethylfluorescein diacetate (CMFDA) (Steinberg et al. 2011). Prior to staining, the cryoprotectant should be diluted by transferring the thawed cultures into 9 ml of sterile medium and by incubating for 24 h to give a more accurate viability index, avoiding damaged cells that still retain metabolic capabilities (Day and Harding 2008; Day et al. 2016). Viable cells will appear green, whereas non-viable cells will turn red or colourless. Alternatively, carboxyfluorescein diacetate succinimidyl ester (CFSE) (Day et al. 2016), erythrosine (Tessarolli et al. 2017), or trypan blue (Shah et al. 2022) can be used. Viability is expressed as the percentage of algal cells that fluoresce compared to the total number of cells observed under a microscope (Johnstone et al. 2002). Viability assessment by direct observation of colonies can also be performed following Day and Brand (2005) or Day and Harding (2008). Note that a slow algal growth rate, low viable cell density, and photoinhibition may hinder the reestablishment of a viable algal culture (Day 2007).

Lichens

As with fungi, the thawing process for lichens should take place quickly in a water bath at 37 °C. Subsequently, symbionts should be cultured *in vitro* without cryoprotectants (Banciu and Cristian 2015), whereas thallus fragments can be cultured outdoors (Honegger 2003). Viability tests are performed using the FDA method. The germination test is expressed as the percentage of cells that germinated (Honegger 2003).

Benthic algae

Samples should be quickly warmed in a water bath at 40 °C. As soon as the ice has melted, thalli should be washed only once in culture medium or transferred to ice-chilled sterilised seawater for 30 min to remove any remaining cryoprotectant (Heesch et al. 2012; Lee and Nam 2016; Visch et al. 2019). Thalli should then be transferred onto Petri dishes for incubation. Strains are considered viable when regrowth is observed under a light microscope (Heesch et al. 2012; Barrento et al. 2016). Erythrosine staining can also be used to check for the viability of vegetative cells. If cells do not absorb the dye, they are considered alive. If they are dyed red, they are considered dead. Gametophytic thallus viability is determined by dividing the number of non-dyed viable cells by the total number of cells (Lee and Nam 2016).

Spore viability can be assessed either by using FDA staining or by recording the percentage of spores that develop into gametophytes (Bhattarai et al. 2007; Zhang et al. 2007). A description of other assay methods can be found in Yang et al. (2021).

Plants

Plant tissues and cell lines are usually warmed in a water bath at 40 °C until completely thawed and cultured in media containing antioxidants to avoid tissue browning (Schumacher et al. 2015; CPC 2019). Cryovials containing seeds should be placed for thawing at room temperature (ca. 20 °C) in a box with silica gel for ca. 20 min, followed by rehydration at 100% for up to 24 h (Ballesteros and Pence 2017; Ballesteros et al. 2021). Embryos and embryo axes should be thawed in a water bath at 36–40 °C for 1–5 min (Normah and Makeen 2008) and cultured *in vitro* to assess survival and growth (Ballesteros and Pence 2019). Ballesteros et al. (2021) provide another thawing protocol for embryonic axes using a Ca/Mg solution. Note that the ideal recovery culture medium for embryos should suppress the production of free radicals (Normah and Makeen 2008). Anthers and pollen can equally be warmed at room temperature for 10–60 min, depending on the species (Ganeshan et al. 2008).

Material that has formed roots can be further grown in field genebanks, or it can be maintained as tissue cultures to assess plant regeneration and genetic fidelity (CPC 2019).

Staining and *in vitro* germination are the most common methods for assessing viability in plants.

Fern spore viability assessment

Vials containing spores should be warmed at room temperature for 20–40 min. Spores are considered viable when the outer wall of the

spore has ruptured and the rhizoid or the first chlorophyllic cell has emerged. A second assessment to calculate the laminar development percentage can take place during the gametophyte transition from one-dimensional filamentous growth to two-dimensional laminar growth.

Spore germination and germination speed can be initially checked daily and then every three days after the 10th day, for a total of 30 days (Ballesteros et al. 2006). The easiest way to assess viability of fern spores is by germinating 100 spores, randomly selected, for a determined number of days (e.g., 10 days for green spores and 25 days for non-green spores) to obtain the germination percentage (Ballesteros et al. 2006; Ballesteros and Pence 2018). Note that the germination media may need to be optimised for each species and can affect the viability assessment results (Pence 2008). Other indirect biochemical assessments are also available and can be revised in Galán and Prada (2011).

For production and acclimatisation of gametophytes and sporophytes in soil, follow Ballesteros and Pence (2018) and Ibars and Estrelles (2015).

Pollen viability assessment

Prior to testing, pollen should be slowly rehydrated to avoid damage from excessively rapid water intake (Towill and Walters 2000). Pollen viability highly differs within and among crop species, hence tests should be standardised for each species/variety (Sidhu 2019). Note that pollen can be viable by being alive and metabolising, but this does not necessarily mean that it can still germinate or fertilise an egg (Towill and Walters 2000). The best choice is to run a combination of viability tests for indications of the pollen´s ability to germinate and grow (Impe et al. 2020).

Pollen stainability includes the use of Triphenyl Tetrazolium Chloride (TTC or TZ) and FDA. Pollen viability percentage is determined by dividing the number of viable pollen grains by the total number of pollen grains. Usually, the quantification of stained/non-stained pollen is done manually, but this task is laborious and time-consuming (Tello et al. 2018). Novel automated approaches have been conceived to assist in this task and they can be applied to different taxa. Ascari et al. (2020) established a procedure based on fluorescence microscopy to assess pollen grain automated images, whereas Tello et al. (2018) developed PollenCounter, an open-source tool for the high-throughput phenotyping of pollen viability. The open-source software ImageJ (US National Institutes of Health) is also used for calculating pollen germination frequency (Smith and Wallace 2020).

In vitro germination comprises pollen tube growth measurements and it requires taxon-specific liquid or solid media (Impe et al. 2020). Pollen grains are considered viable when tube lengths are longer (>1.5×) than the grain diameter (Costa et al. 2020; Anushma et al. 2018). *In vitro* germination assessment can be performed using the hanging drop culture, the sitting drop culture, the suspension culture, or the surface culture method, as described in Gowthami et al. (2019). Refer to Costa et al. (2020), and Impe et al. (2020) for detailed protocols, and to Tushabe and Rosbakh (2021) for a compilation of pollen germination media.

In vivo pollen germination and seed/fruit set percentage methods are other alternatives to assessing pollen viability, although they may be more time-consuming (Gowthami et al. 2019).

Seed viability assessment

Viability tends to decline with time, regardless of the storage conditions, and therefore viability assessments should be carried out periodically (FAO 2014). Furthermore, seeds of different species and even seed lots of the same species may vary in their longevities under standard conditions (Probert et al. 2009; Colville and Pritchard 2019). Note that seeds can be considered viable by germinating but they will not necessarily develop into a viable seedling (Filip Vandelook, pers. comm.).

Ideally, a germination viability test should be performed before storage, and no later than 12 months for orthodox seeds or 3–4 months for recalcitrant seeds after collection. Preferably, 100–200 seeds are used for testing, but the viability

sample size will depend on the accession size (FAO 2014). Reliable results can already be obtained by using 3 × 25 seeds for one testing condition (Filip Vandelook, pers. comm.). Monitoring should take place at least every ten years for base collections and every five years for active collections (MSBP 2015). If the viability rate lies below 85%, regeneration or recollection should be implemented (Engels and Visser 2003; MSBP 2015). A lower threshold (70%) can be set for some landraces and wild/forest species that cannot achieve viability rate of 85% or higher. While international standards for seed banking (FAO 2014) are mainly tailored to crop species in genebanks, it should be remembered that the diversity encompassed by all seed plants is dramatically greater; and processes and standards will probably need to be adapted and relaxed to accommodate wild species preservation in seed banks (Hay and Probert 2013).

RECOMMENDATION
If plant species (e.g., *Bromus* spp.) are susceptible to fungus infection, seeds should be immediately sown in sterilized potting soil, where fungi can be suppressed.

Germination tests of excised embryos and embryonic axes should be assessed by the root production rather than shoot development, as the latter will not occur (FAO 2014).

While the germination test is usually the method of choice for seed viability assessment, vital stains (e.g., TTC) can be informative in species where dormancy is difficult to break (Gosling 2003). TTC is also used to assess the viability of embryos and embryo axes (Normah and Makeen 2008).

RECOMMENDATION
As TTC methods are of limited reliability, they should be preferably used in combination with other methods.

All data regarding germination abnormalities, and viable:inviable seed rates should be recorded to determine whether deterioration is occuring (FAO 2014). Ballesteros et al. (2020) provide a list of gene-banking strategies for maintaining germplasm viability and extending the longevity of seeds.

Cultured plant cell viability assessment

Different methods can be applied to cultured plant cells:

- FDA. The solution fluoresces green under UV in viable cells, whereas dead or damaged cells remain unstained.
- Evan´s blue staining. In this case, inviable cells are stained. This test usually complements the FDA test.
- Phase contrast microscopy is used to assess the presence of a healthy nucleus (Bhojwani and Dantu 2013).
- Micrografting of cryopreserved material.

Plant material that has been maintained for more than ten years in culture is susceptible to somaclonal variations. Hence, these accessions can be grown in the field or a greenhouse for an integrity check, including morphological, cytological, and molecular characterisations (Panis et al. 2020).

Animals

A wide range of thawing conditions are applied to testicular tissue, sperm, and somatic cell suspensions from different taxa. Some amphibian protocols include benchtop thawing or unheated tap water for warming cryovials as soon as they are removed from the LN2

tanks (Browne et al. 2019). However, most thawing protocols are performed by immersing the vials–preferably using tube racks–in a water bath at temperatures ranging between 30 °C to 70 °C, with immersion times from 6 s to 120 s (Klaus et al. 2016; Anel-Lopez et al. 2017; Brito et al. 2017; Browne et al. 2019; Campbell et al. 2020; Daily et al. 2020; Gavin-Plagne et al. 2020; Andrae et al. 2021; García-Salinas et al. 2021; Strand et al. 2021). Note that slow thawing may cause damage to cells (Brito et al. 2017). Furthermore, it has been documented that sperm from fish and amphibians may remain unharmed after warming but no longer capable of activating (Browne et al. 2019). On the other hand, rehydration of sperm previously lyophilised can be completed by adding sterile ultra-pure water into the ampuole (Keskintepe and Eroglu 2015; Anzalone et al. 2018).

The use of ultra-rapid infrared laser warming is an alternative for thawing of oocytes and larvae/embryos because it reduces the chances of ice formation by heating the samples rapidly and uniformly (Khosla et al. 2017; Daly et al. 2018; Gallichotte et al. 2021).

A small portion of germplasm should be periodically thawed and checked for viability, at least once every ten years (FAO 2012).

Oocyte and somatic cell viability assessment

Viability of oocytes and somatic cells can be assessed using trypan blue vital staining (Martínez-Páramo et al. 2016; Siengdee et al. 2018; de Oliveira et al. 2021) or PI for cell-counting using either flow cytometry or a hemocytometer chamber under a microscope (Siengdee et al. 2018; Gavin-Plagne et al. 2020). Viability rate should be calculated according to this formula: (number of live cells/total number of counted cells) × 100 (de Oliveira et al. 2021). Proliferative activity and adhesion measurements are also performed for somatic cells using real-time cell analysis (e.g., xCELLigence, Countess) (Gavin-Plagne et al. 2020; Strand et al. 2021). Cell growth can also be assessed by daily quantifying the population doubling time (PDT) (for details, see Borges et al. 2020). Other viability assessments include the measurements of ROS (reactive oxygen species, i.e., to assess oxidative events) and mitochondrial membrane potential ($\Delta\Psi m$, parameter that controls respiratory rate and ATP synthesis) apoptosis levels (Gerencser et al. 2012; Praxedes et al. 2021; Divakaruni and Jastroch 2022; Murphy et al. 2022). Karyotype analysis can also be carried out to gauge viability (Fukuda et al. 2012).

Sperm viability assessment

Different parameters are used to assess sperm quality, including velocity, activation and motility of spermatozoa, membrane integrity, sperm concentration, DNA integrity, acrosome integrity, as well as –dependent on the availability of oocytes–fertilisation and developmental ability (Browne and Figiel 2011; Anel-Lopez et al. 2017; Cardoso et al. 2020; Browne et al. 2019). Van der Horst (2021) provides a review about high-quality sperm assessment methods in wildlife species and their role in the assessment of cryopreservation procedures.

Sperm concentration

Sperm concentration is determined using a Trypan blue dye and counting with a haemocytometer. It is usually applied to cell stocks (Daily et al. 2020). Sperm morphology can be examined by fixing samples in 10% formol-saline solution in a 1:1 ratio and using a phase contrast microscope (Brito et al. 2017).

Velocity, activation and motility

These aspects can be assessed by using phase contrast microscopes, or a computer-assisted sperm analysis (CASA) together with a plug-in for the ImageJ software (Brito et al. 2017, Browne et al. 2019). However, CASA may not be suitable for small sample volumes, nor for

short motility timeframes of many aquatic species. Instead, using microfluidic platforms to activate sperm is recommended (Hagedorn et al. 2019).

Plasma membrane integrity (PMI)

PMI is tested using a combination of the fluorochromes SYBR-14 and PI (e.g., live/dead sperm viability Kits) and evaluated under a fluorescence microscope. If spermatozoa stain green due to SYBR-14, they are alive; but if they stain red due to PI, it will mean that the membrane has lost its function and the cells are damaged. The percentage of viable cells is then recorded (García-Salinas et al. 2021). The use of eosin/nigrosin stain is also possible (Brito et al. 2017). Note that vital stains may not be reliable when assessing spermatozoa in spermatophores (Browne et al. 2019). Additionally, DAB staining (3.3′ diaminobenzidine) and flow cytometry are used to examine sperm mitochondrial activity (Brito et al. 2017; Martínez-Páramo et al. 2016; Gallichotte et al. 2021).

DNA integrity or DNA fragmentation (SDF) and apoptosis

The degree of SDF and apoptosis are examined using a TUNEL assay (e.g., *in situ* cell death detection kit) under a fluorescence microscope. Spermatozoa with normal DNA dye blue, due to the Hoechst 33342 stain, whereas apoptotic spermatozoa and those with fragmented DNA stain red. The final percentage of cells containing fragmented DNA can be calculated by dividing the number of red cells by the number of blue cells (Cardoso et al. 2020; Gallichotte et al. 2021). Alternatively, the comet assay (single-cell gel electrophoresis assay) can be used to detect DNA damage after cryopreservation (Loi et al. 2008; Martínez-Páramo et al. 2016).

Acrosome integrity (AI)

AI can be assessed using either a modified Fast Green/Rose Bengal stain protocol (Brito et al. 2017), or the PI stain protocol together with peanut agglutinin (PNA) (Cardoso et al. 2020). If cells are negative for both PI and PNA, they are considered viable cells with intact acrosomes (Cardoso et al. 2020).

Other tests

Other sperm quality assessment tests include cell metabolism damage, peroxidation events and proteome analyses (Martínez-Páramo et al. 2016). In insects, reproductive (e.g., fertility, hatching), phenotypic, physical (e.g., flight ability), and molecular tests (e.g., gene expression) can also be performed on the progeny produced with the tested sperm (Gallichotte et al. 2021). More detailed information regarding viability assessments and sperm morphology in marine invertebrates can be found in Lewis and Ford (2012).

Environmental samples

So far, only Rain-Franco et al. (2021) have suggested a cryopreservation and thawing protocol for microbial communities found in water samples. Thawing should take place at room temperature for 30 min, after which the samples are added to a culture medium for incubation at a temperature similar to the temperature the sample was originally exposed to in the field. Sample assessment is carried out using flow cytometry to count cell numbers.

CHAPTER 8
DNA

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Introduction

DNA data have been used to inform, among others, the identification of hidden species, detection of taxa and individuals, hybridisation, population genetic analyses, and phylogenetics (Andrews et al. 2018). DNA can be isolated from any organism and any tissue/pseudotissue with different degrees of success (Tamari and Hinkley 2016). Hence, it is important to choose the right part of the organism to obtain good DNA quality and sufficient quantity. For instance, DNA from young leaves of plants, spores of fungi, sporocysts of myxomycetes or reproductive structures of lichens are generally considered optimal for DNA analyses (Buyck et al. 2010; Tamari and Hinkley 2016). Note that DNA extraction success will also strongly depend on the preservation method (e.g., freezing, Lugol's solution), cell lysis protocol and DNA isolation method.

Recommendations for DNA handling

Before starting and during any DNA extraction procedure, contamination risks should be minimised by following these recommendations:

- Filter tips should be used, where needed, to avoid possible contamination from the pipettes.
- Gloves should be worn and discarded periodically and if contaminated.
- Ideally, there should be separate rooms for pre-PCR and post-PCR procedures.
- Benchtops should regularly be decontaminated using e.g., bleach, TriGene, Eliminaze, or Distel, as should be the equipment e.g., by flame or bead heater.
- Handling techniques should be optimised (e.g., pipetting).
- If possible, procedures should take place under a laminar flow.
- All samples and reagent vials should be kept closed when not in use.
- Ideally, plastic material, water and solutions should be RNase- and DNase-free.

Ancient DNA

Ancient DNA (aDNA) analysis is a technique that can provide genetic information on plant and animal remains from the Pleistocene and Holocene. The recovery of low-coverage genome data from, e.g., a 700,000-year-old horse (Orlando et al. 2013), from a million-year-old mammoth (van der Valk et al. 2021), or from a 2-million-year-old ecosystem (Kjær et al. 2022) has elucidated that the temporal limit of ancient DNA can be pushed back further than the previously old suggested 100,000 years (Lindahl 1993).

aDNA requires destructive sampling, which can diminish the specimen research/display value or may imply loss of the specimen. Hence it is important to make informed decisions about maximising DNA recovery before the sampling process starts (Freedman et al. 2018; Pálsdóttir et al. 2019).

Ancient DNA principles

Working with aDNA requires a specialised laboratory that is physically isolated from any post PCR area and other laboratories, with an independent HEPA-filtered air source and, ideally it should include separate areas for sample preparation, DNA extraction and sequencing library preparation/PCR setup (Kurushima et al. 2012). It should follow rigorous protocols to deal with contamination and problems with

authentication (Shapiro and Cooper 2003). All equipment should be periodically sterilised with bleach and DNA Away wipes (Molecular Bio-Products) or similar products, and sterile personal protective equipment should be used including full isolation gowns, gloves, face shields, facemasks, hair coverings, and shoe coverings (Kurushima et al. 2012). In addition, PCR products from other lab facilities should never be transferred to the aDNA lab. Refer to Fulton (2012) for more details on how to set up an aDNA lab, and to Gilbert et al. (2005), Llamas et al. (2017), and Orlando et al. (2021) for more detailed guidelines on aDNA research rigour.

Sufficient amounts of authentic endogenous DNA are difficult to retrieve, first because there exists an amplification bias for undamaged modern DNA during PCR, even when aDNA might be found in higher concentrations (Shapiro and Cooper 2003), and second because oxidation and hydrolytic processes cause DNA degradation, leading to depurination (Pruvost et al. 2007; Rizzi et al. 2012).

RECOMMENDATION
Consider beforehand what type of extraction, purification, and library protocols are going to be used, as retrieval of aDNA, including length and yield, will depend on these. Lab protocols should be adequately optimised for each type of sample.

DNA extracts can be enzymatically treated (e.g., uracil-DNA glycosylase) prior to sequencing library creation to remove uracil residues that resulted from DNA damage (Pruvost et al. 2007; Kehlmaier et al. 2017). DNA libraries can be sequenced directly, or alternatively, enriched for target sequences utilising bead-based protocols (e.g., commercial MyBaits target capture kit, Arbor Biosciences) or SNP chip arrays (e.g., Axiom Genome-Wide Human Origins 1 Array). The latter approach is of particular advantage when the target DNA is present in low amounts in a mixture of environmental components–as in the case of very poorly preserved samples or if targeting a specific pathogen–or when dealing with complex genomes (Hofreiter et al. 2015; Meyer et al. 2017; Orlando et al. 2021). Whole-genome shotgun sequencing approaches, on the other hand, can evenly cover the entire metagenome (Orlando et al. 2021) and with the decrease in sequencing costs and methodological improvements in aDNA recovery (e.g., Pinhasi et al. 2015), can offer a more efficient approach. While target enrichment can gather data from very degraded samples, it is subject to bait-design reference bias and can limit the amount of data recovered from better-preserved samples by overlooking non-targeted SNPs (Der Sarkissian et al. 2015; Orlando et al. 2021).

Because of the fragmented nature of aDNA, short-read sequencing such as Illumina is particularly indicated. Library preparation protocols should be especially developed or adapted because commercially available approaches may discard short fragments after clean-up steps, reducing the recovery of aDNA (Kapp et al. 2021). These protocols should include intermediate purification steps to retrieve high-quality aDNA libraries, unless single-tube approaches are used (Carøe et al. 2018). Single-stranded (ssDNA) library preparation protocols are particularly suited, for instance, for quaternary samples, formalin-fixed or paraffin-embedded tissues because they can incorporate a higher proportion of degraded DNA into sequencing library molecules (Henneberger et al. 2019; Kapp et al. 2021; Orlando et al. 2021) but can also be applied to younger museum material.

RECOMMENDATION
Negative controls (blanks) have to be included during grinding, extraction, and PCR to monitor contamination. Furthermore, replication of DNA procedures in a secondary lab are no longer required for aDNA data validation.

Various aDNA extraction protocols for different types of samples can be found in the field collection section under "historical museum samples" and further protocols in the "further reading" annex.

DNA extraction of fresh samples

Before proceeding with the extraction of DNA, it is highly recommended to use subsamples instead of the whole collected material. It is also advisable to test *a priori* different extraction protocols, using a small number of samples in order to find the most appropriate one for a particular project (Weigt et al. 2012; Martincová and Aghová 2020). The most suitable extraction method will depend on the starting material, sample size, expected DNA concentration and purity, and price (Martincová and Aghová 2020). Note that commercial kits that have been designed for specific starting material can be adapted to fit other types of samples (Sellers et al. 2018).

Protists and microalgae

Any stage of the life cycle of fungus-like forms and other protists, including spores, amoeboflagellates, plasmodia, sporocysts, microcysts, sclerotia and fruiting bodies, is suitable for DNA isolation (Walker et al. 2017; Wrigley de Basanta and Estrada-Torres 2017).

Ideally, bead-beating protocols together with freeze-thaw lysis should be used to break firm cell walls of protists and increase the DNA yield (Mäki et al. 2017; Martín et al. 2020). Samples that have been preserved in ethanol should first be treated to evaporate the ethanol, prior to DNA extraction (Schoenle et al. 2021). If DNA is isolated from protist cultures (ca. 2 ml), these should first be centrifuged to concentrate the culture, and the supernatant removed (Balzano et al. 2012; Schoenle et al. 2021). Sediment samples should be prewashed to improve PCR success (Schoenle et al. 2021). The phenol-chloroform DNA extraction method is commonly employed (Altermatt et al. 2015), yet some commercial kits can also be used, e.g., Quick-gDNA Mini Prep Kit (Zymo) for protistan strains or the DNeasy PowerSoil DNA isolation kit (Qiagen) for soil/sediment samples.

Medinger et al. (2010) and Auinger et al. (2008) provide protocols for sample preparation and DNA extraction from protists preserved in Lugol's solution, as well as DNA extraction from single-cell isolation. Ciobanu et al. (2021, 2022) also suggest a protocol for single-cell isolation from environmental microbial samples.

A protocol including microalgal sample preparation for DNA extraction using in-house filters (e.g., Sterivex) is provided by the Nansen legacy (2021). Methods for freshwater microalgae can be found in Eland et al. (2012). Protocols have also been optimised for diatoms (Annunziata et al. 2021), *Prototheca* algae (Jagielski et al. 2017), and green algae (Stark et al. 2020; Kunyalung et al. 2021; Romac 2022). DNA quality of phytoplankton should be assessed before extraction to estimate the water dilution factor needed to achieve 25 ng DA/µl for PCR and subsequent high-throughput sequencing (Bailet et al. 2019).

RECOMMENDATION
Do not fix microalgae in alcohol because DNA yields will be reduced considerably.

Microfungi

Some initial preparations need to be carried out, depending on the type of material to be used for DNA isolation. If cultures are not completely pure, plugs (ca. 8 mm in diameter) should be cut from the periphery of a colony, inoculated in another medium, and incubated for 5–10 days (Simões 2013). Mycelia can be harvested (ca. 10–40 mg) with a sterile scalpel into a 1-ml clean Eppendorf tube and homogenised/grinded using a lysis buffer and either sterile stainless-steel beads or a mortar to break the chitin cell walls (Akinsanmi et al. 2017; Crous et al. 2019; Martín et al. 2020; Serrano-Jamaica et al. 2021). Otherwise, mycelia should be allowed to dry, then to be frozen until further

processing (Simões 2013; Martín et al. 2020; Serrano-Jamaica et al. 2021). Monoconidial and ascospore cultures can also be used to obtain fungal mycelia (Crous et al. 2019). Spores and microcysts can be physically disrupted using a sterile pestle, glass beads or sand (Walker et al. 2017). To break hard pieces of sclerotia or wood, soil samples (ca. 10 g) should be homogenised using, for instance, a cell disruptor (e.g., FastPrep System, Thermo Savant) (Lievens et al. 2012; Penton et al. 2016). Sonication techniques can also be used to break fungal cell walls to release cell contents (Pilo et al. 2022).

For plant pathogenic organisms, either a single sorus from a strain can be excised from the infected host tissue (Feau et al. 2009) or both plant and pathogen DNA can be isolated together (Datlof et al. 2017). The latter has the advantage of requiring no culture, but DNA from other fungal species may be extracted alongside the target DNA (Capote et al. 2012). Representative plant tissue (ca. 1 g) should be carefully selected, as it should include the pathogens of concern. Pooling several samples from different plant parts into one extraction is also possible, but they can also be processed independently and pooled later (Lievens et al. 2012). Homogenisation of infected plant tissues can be achieved by using enzymes (e.g., chitinase) or by mechanical lysis (e.g., grinding in LN2), or by a combination of both methods (Capote et al. 2012). Note that some secondary compounds may be released, so the addition of PVP or CTAB into the extraction buffer may be necessary (Capote et al. 2012).

Protocols for mycorrhizal fungi can be found in Allen et al. (2003) and Manian et al. (2001). DNA mini prep protocols from mycorrhizal samples, lyophilised tissue and fresh fungal tissue can be found on the **Vilgalys Lab website**.

High molecular weight DNA from pure cultures can be extracted using different kits, such as the Promega Wizard Genomic DNA purification kit (Akinsanmi et al. 2017), the UltraClean Microbial DNA Isolation Kit (MoBio) (Crous et al. 2019), the PowerPlant DNA isolation kit (MoBio), or the DNeasy Plant Mini Kit (Qiagen) (Martín et al. 2020). The CTAB method (Serrano-Jamaica et al. 2021) can be used for stone chip and fungi-tape samples (Cutler et al. 2012). DNA from fungal pathogens can be isolated using the DNeasy Plant Mini kit (Qiagen) (Feau et al. 2020) or the FastDNA SPIN kit (MP Biomedicals) (Da Lio et al. 2018), whereas DNA from soil samples can be extracted using the PowerSoil DNA isolation kit (MoBio) (Lievens et al. 2012). Further protocols for pathogenic fungi are provided in Gupta et al. (2013) and Smith et al. (2020). Different protocols for DNA extraction from filamentous fungi are provided by Conlon et al. (2022). If only DNA barcoding or microsatellite amplification is intended, then the NaOH extraction protocol by Osmundson et al. (2013) may be used. Several DNA extraction protocols are described in Narayanasamy (2011).

Usually, DNA should be purified but this can be time consuming. One alternative to avoid this step is to load the DNA extract onto FTA cards, which can be stored at room temperature for years (Capote et al. 2012).

Macrofungi

Most protocols for extracting DNA from mushrooms require grinding and purification steps (Hosaka and Uno 2011). Around 40 mg of dried fungus can be ground in a centrifuge tube containing glass beads (Wang et al. 2017). The CTAB method is commonly used, with some modifications for fungi with a high polysaccharide content (Huang et al. 2018). Izumitsu et al. (2012) developed a protocol that combines microwaving and cooling of fungal samples that can also be used for screening genetic transformants of fungal cultures. Dry samples can be hydrated with 5% KOH to be crushed on FTA paper for subsequent DNA isolation (Telleria et al. 2014). Further DNA extraction protocols for macrofungi can be found in Dentinger et al. (2010), Eberhardt (2012), and on the **Vilgalys Lab website**.

Lichens

Prior to DNA extraction, fresh lichen fragments (for thalli ca. 10 mg or for apothecia ca. 3 mm^3)

can be washed in 0.85% NaCl and then in sterile water (Dal Forno et al. 2022). DNA can be obtained by first breaking lichen tissue with a bead-beater until a fine powder is formed. One method involves putting the lichen tissue into a vial containing glass beads, which is then frozen in LN2 prior to disruption. Refer to Park et al. (2014) or to Ivanova et al. (2011) for a complete protocol. There are multiple kits that can be used for lichen extraction, including the DNeasy PowerSoil kit. Further details can be found in Cubero and Crespo (2002). Note that commercial kits for DNA isolation can be used to obtain high DNA quality with less than 10 mg of starting material (Martín and Winka 2000).

Alternative methods include prewashing the lichen tissue with 50 µl of acetone for up to half an hour; the acetone can then be recovered and dried down for chromatography, and the lichen tissue left to air dry for ca. 10 minutes prior to DNA extraction. This removes some of the secondary chemistry that might interfere with PCR downstream. The lichens can then be ground using tungsten beads in a mixer mill, or with mini-pestles and a pinch of acid-washed sand, and the DNA can be extracted with CTAB or with Qiagen's Plant DNAeasy mini kits (e.g., DToL protocols). An unrelated, straightforward protocol that can give good results with lichens is the REDExtract-N-Amp Plant PCR Kit (Rebecca Yahr, pers. comm.).

Benthic algae

For DNA analyses of macroalgae, small pieces of clean thalli (i.e., free of epiphytes) (ca. 4–5 cm diameter or 4 g) can be cut, snap frozen in LN2 to break rigid cell walls, freeze-dried and ground into a fine powder using a tissue lyser. Around 20 mg of ground material usually suffice for DNA extraction. Samples can then be stored at -20 °C or -80 °C to await further processing (Fort et al. 2018; 2021). Macroalgae, like plants, also contain polysaccharides and phenolics that can inhibit PCR; hence, a purification step should be included in the process (Fort et al. 2018). Various protocols based on column-based DNA extraction kits, Chelex, CTAB, and phenol/chloroform are available (Joubert and Fleurence 2005; Hoarau et al. 2007; Greco et al. 2014; Prasanthi et al. 2020). A compilation of protocols can be found in Wilson et al. (2016). Fort et al. (2018) established a protocol using magnetic beads and the Macherey-Nagel NucleoMag plant kit that yields high amounts of DNA. Shin et al. (2021) validated a boiling DNA extraction method for different macroalgae, but it should not be applied to species with high polysaccharide content.

Plants

Plant tissue, wood and seeds should be ground prior to DNA extraction to break up the cell walls (preceded by an optional LN2 freezing step), using sterile sand as an abrasive. To avoid the production of frictional heat, grinding is best done over ice. For large seeds, the endosperm should be removed prior to grinding, and the embryo used for DNA extraction (Gemeinholzer et al. 2010). Both fresh and dried material can be homogenised in a mixer mill or tissue lyser before DNA extraction following Neves and Forrest (2011). The resulting fine powder can be a source of contamination when handling tubes and opening lids, so if dealing with multiple taxa, samples from the same genus or species should not be placed in consecutive tubes, so that contamination can be identified more easily when analysing DNA sequences (Neves and Forrest 2011). Plant material can also be homogenised in a sorbitol wash buffer. This step helps to remove compounds such as polyphenolics and polysaccharides without affecting the DNA (Štorchová et al. 2000).

Several DNA extraction protocols containing a variety of buffers, as well as different commercial kits (e.g., Qiagen DNeasy Plant Maxi and Mini Kit, PowerPlant Pro kit, Macherey-Nagel's Nucleospin, Tiangen plant genomic extraction kit) have been developed, not only to isolate genomic DNA but also to eliminate further primary and secondary compounds, which can hinder downstream applications (Hodkinson et al. 2007; Ivanova et al. 2008; Gemeinholzer et al. 2010; Abdel-Latif

and Osman 2017). It is important to understand the role of each chemical used in the extraction procedure, as their concentrations are usually modified and adjusted for specific taxa. Hence, there is no single protocol that can be applied equally to all taxa. Note that an additional DNA purification step should follow, if caesium chloride gradients, phenol-chloroform, phenol-ethanol, or dialysis are used (Hodkinson et al. 2007).

Inexpensive CTAB extraction methods are the most widely used, with modifications to optimise DNA isolation from recalcitrant plants (e.g., Cactaceae, or cacao) (Hodkinson et al. 2007; Spooner and Ruess 2014). A summary of DNA extraction methods can be found in Weising et al. (2005) and Tamari and Hinkley (2016). Further information and protocols are listed at the end of this section. An improved protocol for genomic DNA extraction from bryophytes can be found in Pandey et al. (2019). DNA from ethanol-preserved plants can be extracted following Bressan et al. (2014).

Animals

Before DNA extraction, some preliminary steps should be carried out depending on the sample preservation method: 1) ethanol has to be removed from alcohol-preserved tissues by air-drying or oven-drying (below 60 °C) until totally evaporated (Nagy 2010), 2) large and desiccated tissue should be covered with buffer (e.g., PBS) and incubated in a shaker (21 °C) for several hours or overnight for rehydration, and 3) frozen tissue should be equilibrated to room temperature. Samples on FTA cards, fresh tissue and blood can be immediately used for DNA extraction (Australian Museum 2019).

The Qiagen DNeasy Blood and Tissue commercial kit is the most widely used method for DNA extraction (Chakraborty et al. 2020), along with other commercial silica membrane-based kits. However, Ivanova et al. (2006) and Peñafiel et al. (2019) developed manual low-cost, and high-quality DNA extraction protocols that can be used for different tissues (e.g., cell suspensions from skin swabs, blood, muscle) from different animal taxa. Traditional methods include phenol-chloroform-isoamyl alcohol and SDS (Akinwole and Babarinde 2019), or the use of Chelex resin methods (Singh et al. 2018; Li et al. 2019), which have been successfully used for diverse taxa. Note that DNA quantification is not precise for Chelex extracts due to the presence of cellular elements (Seah et al. 2020, see supplementary material). For the same reason, DNA extracted with Chelex does not lend itself well for long-term archival in biobanks.

Several protocols have been specifically developed or optimised for insects (Cruaud et al. 2019; Facchini et al. 2018; Heavens et al. 2021; Marquina et al. 2022), other small arthropods (Lienhard and Schäffer 2019), fish (Gui et al. 2022), and for non-invasive sampling (Ferreira et al. 2018; Biswas et al. 2019; Bourgeois et al. 2019; Natesh et al. 2019; Buzan et al. 2020; Khan et al. 2020; Sarabia et al. 2020; Seah et al. 2020; Olah 2021; Lefort et al. 2022; Schmerer 2022). DNA from gastropods and bivalves, which produce excessive slime (mucopolysaccharides), should be extracted preferably with a CTAB method modified by Chakraborty et al. (2020). DNA extraction protocols for animal cells can be found in Jain et al. (2020).

Parasites

Identified specimens may be pooled per species (e.g., between 5–10 individuals or 30–90 individuals for gregarines) (Clopton 2009). Before DNA extraction, ethanol should be removed from samples either by evaporation at room temperature or by soaking them in Tris-EDTA buffer overnight (Bray et al. 2020). Different DNA extraction kits can be used, such as the DNeasy blood and tissue kit (Yuan et al. 2016), MasterPure Complete DNA and RNA Purification kit (Epicentre Biotechnologies) (Rueckert et al. 2011; Wakeman and Leader 2012), or PureLink genomic DNA mini kit (Invitrogen) (Clopton 2009). DNA extraction methods for single individuals can be found in Sakai (2010), Blaxter (2014) and Sloan et al. (2021).

Environmental DNA

Lear et al. (2018) provide some recommendations and step-by-step protocols for eDNA from different community/taxa types regarding sample pre-processing, DNA extraction, storage, amplification, and sequencing. Lever et al. (2015) and Sellers et al. (2018) optimised DNA extraction protocols suitable for air, water, soil, sediment and tissue samples, whereas Hermans et al. (2018) identified the DNeasy PowerSoil as the best solution for isolating DNA from any sample type. In the section below more protocols are mentioned which are suitable for different sample types. Note that the DNA extraction method will influence not only the quantity and quality of the DNA, but also the resulting observed community composition of the sample (Zielińska et al. 2017; Iturbe-Espinoza et al. 2021).

RECOMMENDATION
PCR inhibition can be caused by humic acid and tannin compounds, found in turbid water, soil, and sediments, which can inactivate the DNA polymerase, causing false negatives. It is crucial to test for this, either by adding internal positive controls (IPS), internal amplification controls (IAC), or performing a droplet digital PCR. The impact of inhibition can also be minimised, by adding bovine serum albumen (BSA) to PCR reactions, or by using clean-up kits (e.g., Zymo or Qiagen) (Harper et al. 2019). eDNA extracts and PCR template should not be diluted, since it may result in non-detections.

Water samples

If water has been kept frozen, it should first be thawed at room temperature and mixed to homogenise the sample before filtering (Thomsen et al. 2016).

Open filters should be cut into thin slices (ca. 1 mm) and placed in 2 ml tubes for bead-beating before DNA extraction (Thomsen et al. 2012). Enclosed-capsule filters can be cracked open to remove the filter, which is cut into small pieces (Cruaud et al. 2017). However, it is also possible to add lysis buffers and proteinase K directly onto the housed filters to be incubated. Then the buffer is taken off for subsequent DNA extraction (Anderson and Thompson 2022). Different extraction protocols can be used for open filters, among them commercial DNA extraction kits (e.g., Qiagen DNeasy Blood & Tissue kit), CTAB, or PCI protocols (Shu et al. 2020). Note that phase separation and precipitation methods tend to yield more DNA than silica column methods (Goldberg et al. 2016). However, if the water sample contains humic compounds or sediment, the best option is to use the PowerWater DNA Isolation kit, which already includes an inhibitor removal step (Shu et al. 2020). In addition, the use of TaqMan1 Environmental Master Mix in the PCR mix may help decrease inhibition (Hinlo et al. 2017). On the other hand, the PowerWater DNA Isolation kit is not recommended for eDNA samples that were stored in ethanol, as these samples are not compatible with the kit (Hinlo et al. 2017). Bead-based extraction methods are mainly used for housed filters (e.g., ZymoBIOMICS MagBead kit, NucleoMag kit) (Ushio et al. 2018; Anderson and Thompson 2021).

Optimised DNA extraction protocols for different types of filters can be found in Spens et al. (2017) and in Egeter et al. (2018). Both Djurhuus et al. (2017) and Jeunen et al. (2019) provide comparisons of filters and extraction protocols for metabarcoding studies. Sanches and Schreier (2020) have developed a DNA extraction protocol for estuarine environments. Moreover, a summary of technical protocols for fish eDNA detection, as well as a detailed comparison between eDNA yield, costs, and processing time can be found in Shu et al. (2020). Sellers et al. (2018) developed a modular universal DNA extraction method that can be applied to water, soil, stool, and tissue samples.

Sediment and soil samples

Samples that have been stored frozen should be thawed overnight at 4 °C and then mixed

thoroughly (Laroche et al. 2020; Kirse et al. 2021). Samples that were preserved in ethanol LifeGuard or any other soil preservation solution should first be centrifuged, and the supernatant discarded to avoid compounds interfering with the extraction kits (Bruce et al. 2021). Homogenisation can be performed by adding saturated phosphate buffer, in a 1:1 ratio for 15 minutes (Taberlet et al. 2012; Foucher et al. 2020; Kirse et al. 2021). Note that a shorter time will reduce the eDNA concentration, whereas a longer time will increase humic acids in the buffer (Taberlet et al. 2012). Sample quantity ranges between 0.5 g and 10 g. Usually 2ml of the mixture is centrifuged and the supernatant is used for subsequent extraction (Foucher et al. 2020). Multiple duplicates per sample can also be extracted to increase representation (Bruce et al. 2021; Kirse et al. 2021). Some of the most frequently used commercial kits are the DNeasy PowerMax Soil kit (Qiagen) and the NucleoSpin Soil kit (Macherey-Nagel) (Taberlet et al. 2012; Rota et al. 2020; Foucher et al. 2020; Kirse et al. 2021). Since these kits were mainly developed for DNA extraction from microorganisms, their lysis step is generally omitted when working with extracellular DNA (Taberlet et al. 2012), unless microorganisms are the targeted taxa. Another alternative is to use the Sediment CTAB method (Mitchell and Takacs-Vesbach 2008). It is also possible to add a DNA purification step using the PowerClean Pro DNA Clean-Up kit (MoBio) following the manufacturer's protocol (Kirse et al. 2021), or the MagBind TotalPure NGS (Omega Biotek) (Díaz et al. 2020). Note that the use of commercial kits will allow for more consistent results than the ones given by traditional self-mixed extraction methods (Zielińska et al. 2017).

RECOMMENDATION
DNA extraction techniques should be optimised for each soil type, due to the differences in their physicochemical properties (Zielińska et al. 2017).

Note that macrofauna should be analysed as bulk DNA, microbial communities as direct eDNA extractions, and meiofauna can be analysed using either method (Pawlowski et al. 2022).

A modular method for eDNA extraction from different environments (including water, snowmelt, and airborne samples) has been developed by Lever et al. (2015, see online material as well), including recommendations for optimisation depending on the strata composition. Some protocols are provided by the **CALeDNA** program. A complete overview of DNA extraction from sediments, including protocols, can be found in Capo et al. (2021).

Airborne samples

Ideally, all equipment should be sterilised using UV, 10% bleach, 70% ethanol and ultrapure water between each sample (Clare et al. 2022). Sterivex filters can be cracked open, and the filter removed and cut into small pieces following Cruaud et al. (2017). Otherwise, follow Spens et al. (2017) if extraction should take place inside the casing. Tapes and filters should be ground using beads for five minutes in a bead-beater or using a sterile plastic pestle and vortex agitation for homogenisation (Banchi et al. 2018; Johnson et al. 2021). DNA can be extracted using the Qiagen DNeasy blood & tissue kit, the Qiagen DNeasy PowerPlant Pro DNA Isolation kit or the ZR Fungal/Bacterial DNA MicroPrep kit (Zymo) following the manufacturers' protocols. Volume of ATL buffer may be increased to completely submerge the filter (Clare et al. 2022).

Filters and tapes from the same sampling event can be put together before DNA extraction or pooled at the last step of the DNA extraction protocol respectively (Banchi et al. 2018; Johnson et al. 2021).

Note: Quality control is important to determine sample degradation and inhibitor-removal efficiency (Harper et al. 2019). To accomplish this, standardised non-target DNAs, called exogenous IPCs, can be included at different steps of the preservation

and extraction procedures. For instance, IPCs can be added to the preservative solution, the first step of DNA extraction or to the multiplex PCR mix (Harper et al. 2019; Bruce et al. 2021). If IPC amplification during the qPCR/ddPCR reaction is then unsuccessful or delayed, the sample is inhibited or degraded (Bruce et al. 2021). Validation of assays should be a requirement to lessen uncertainties and limitations associated with eDNA studies, as most of the time it is difficult to know whether a methodology is reliable or not for species monitoring (Thalinger et al. 2021). Validation can also provide information on how a procedure can be modified and can eventually lead to the creation of new standards for future studies (Thalinger et al. 2021). Thalinger et al. (2021) developed an additive five-level validation system that should be used as a standard checklist to ensure that a high performance is achieved. More information can also be found in https://edna-validation.com/.

Storage

DNA quality will depend on the preservation method and duration of storage (Walters and Hanner 2006). Freezing is the most common method for storing DNA (Howlett et al. 2014). Normally, DNA is stored in TE or TBE buffer, and ideally, it should be portioned into two aliquots: one for long-term storage at -80 °C or in liquid nitrogen and, the other one kept as working material at -20 °C (Hodkinson et al. 2007; Knebelsberger and Stöger 2012). However, the costs of maintaining a cold chain (e.g., mechanical freezers, liquid nitrogen tanks) and the need for DNA backup storage have emphasised the demand for room temperature methods (Wan et al. 2010; Fabre et al. 2013; Ivanova and Kuzmina 2013). Room temperature storage requires that some environmental factors, such as water (humidity) and oxygen, are controlled to avoid DNA damage and deterioration (e.g., DNA breaks, mispairs, cross-linked nucleotides) (Howlett et al. 2014; Coudy et al. 2021). If high molecular weight DNA is needed within a relatively short time span, samples should not be frozen and instead should be stored at 4 °C (Daniel Mulcahy, pers. observation), as freeze-thaw cycles can break long strands of DNA (Shao et al. 2012). Refer to Ruvira and Ruiz Arahal (2012) for DNA storage protocols applied at various microbial biobanks.

RECOMMENDATION
Repeated freeze-thaw cycles should be avoided because they will degrade the DNA.

Several lyophilisation or freeze-drying methods have been developed and are commonly used for the long-term storage and transportation of DNA at room temperature (Washetine et al. 2019). It is possible to dry-powder DNA using a freeze drier (Guo et al. 2018). Ideally, dry DNA should be stored in polypropylene vials (Trapmann et al. 2004) with lo-bind coating to reduce DNA adhesion to the tube walls. However, freeze-drying may concentrate buffer salts, and DNA powder can be blown out or moved by electrostatic charges. Dried-powdered DNA has to be resuspended, so it will not be available for immediate use.

RECOMMENDATION
Different factors (e.g., sample type, use, logistics, costs) will determine the choice of storage system, which in turn, must not alter the DNA sample or endanger its integrity.

Commercial stability reagents such as GenTegra (IntegeneX), DNAstable (BioMatrica) or

QIAsafe DNA Tubes (Qiagen), contain a thermo-stable inorganic mineral matrix that forms a coat around the DNA, protecting it from oxidative and hydrolytic processes and microbial activity (Clermont et al. 2014; Muller et al. 2016). The use of preserving agents like trehalose or Tris-buffered PVA (polyvinyl alcohol) has also been recommended (Knebelsberger and Stöger 2012; Ivanova and Kuzmina 2013; Muller et al. 2016). DNA should be placed in tubes or plate wells containing the stability reagents for subsequent drying. DNA should be completely dried either by air-drying in a laminar flow hood, by vacuum drying (e.g., SpeedVac, Thermo-Savant Explorer concentrator), or by using a FastDryer (Clermont et al. 2014; Muller et al. 2016).

Samples can be stored at room temperature with a RH below 50%. If RH is higher, samples should be placed into moisture-barrier seals. QIAsafe DNA Tubes should preferably be stored at -80 °C, rather than at room temperature (Knebelsberger and Stöger 2012). Samples can be recovered by adding water or an aqueous buffer. Keep in mind that the tubes provided with these commercial reagents may still be affected by humidity, as they are not watertight (Clermont et al. 2014). Furthermore, higher temperatures (ca. 60°–70 °C) are still a crucial factor that compromises for DNA quality and should be considered when using any dry storage systems (Ivanova and Kuzmina 2013).

Note that the above-mentioned stability reagents are affected by the selected DNA extraction method, and hence, reagent protocols should be optimised (Frippiat et al. 2011).

RECOMMENDATION
DNA degradation can be assessed using The TapeStation (Agilent) system, as well as SYBR1 Green-based qPCR assays and the standard COmbined DNA Index System (CODIS) Short Tandem Repeat polymorphisms (STR) kits. See Howlett et al. (2014) for further details.

Another alternative, and currently the technically most elaborate option, with good test results, is the use of DNAshells (Imagene), where genomic material is stored in open glass capillaries and encapsulated in watertight, oxidation-proof metal capsules containing an anhydrous and anoxic environment (Clermont et al. 2014; Washetine et al. 2019; Coudy et al. 2021). DNA has to be dried, ideally, in the presence of trehalose to stabilise the secondary DNA structure as well as to improve DNA recovery (Clermont et al. 2014). The storage of slightly degraded DNA in DNAshells can stop further degradation (Clermont et al. 2014; Coudy et al. 2021). Note that the encapsulation process requires professional welding and leak control in a special encapsulation station (Li et al. 2017).

FTA paper cards (e.g., Whatman or CloneSaver cards) are also suitable for storing DNA, especially that from blood, parasites, fungi, plants, or insects (Owens and Szalanski 2005; Martín, pers. comm.). This method is usually preferred over ethanol preservation of unextracted tissue for long-term storage (Borisenko et al. 2007; Eberhardt 2012). However, FTA paper leaves the DNA samples directly exposed to the atmosphere (Coudy et al. 2021). Placing the cards in sealable plastic bags that contain a small amount of an indicating desiccant and storing them in card file cabinets can lessen degradation (Owens and Szalanski 2005; Dentinger et al. 2010). The main advantage of the filter paper is that it offers an easy way for transportation and distribution. Smith and Burgoyne (2004) provide a summary of protocols for processing samples stored on FTA paper according to sample type and end use. Alternatively, FTA paper can be pre-coated with silk (6%) and treated with methanol, which can also help to maintain a high DNA molecular weight (Liu et al. 2017).

Other DNA storage options include the use of 50% glycerol (Schaudien et al. 2007; Röder et al. (2010), earth alkaline salts (e.g., calcium phosphate, calcium chloride and magnesium chloride) (Kohll et al. 2020), and eventually, the DNA-shielding function of the tardigrade Dsup (damage suppressor) protein, which remains to be elucidated (Mínguez-Toral et al. 2020). A general review on storage of DNA is provided by Garafutdinov et al. (2020).

CHAPTER 9
High-Molecular-Weight DNA

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Introduction

International initiatives, such as the Earth BioGenome Project, are aiming to characterise complete genomes of all extant species using long-read sequencing technologies (e.g., PacBio, Oxford Nanopore) (Blaxter et al. 2022; Formenti et al. 2022). Hence, long DNA fragments are crucial, and can only be obtained by having access to high-molecular-weight DNA (HMW, 45–150 kb) and ultra-high-molecular-weight DNA (uHMW, over 150 kb) (Mulcahy et al. 2016; Ryder and Onuma 2018; Dahn et al. 2022).

Although second-generation technologies (e.g., Illumina) have transformed DNA analyses and become standard applications (Kchouk et al. 2017; Slatko et al. 2018), they are based on short-read sequencing and cannot deal with complex genomes that contain repetitive areas; thus, the resulting genome assemblies are often incomplete and fragmented (Kchouk et al. 2017; Dahn et al. 2022). Long-read sequencing or third-generation sequencing, on the other hand, can generate fragments long enough to overlap, despite of having a higher error rate (Rhoads and Au 2015; Amarasinghe et al. 2020; Delahaye and Nicolas 2021).

The performance of HMW DNA is dependent on the sample collection and DNA extraction procedures. From the start during field collection, adequate sample preservation methods have to be used to maintain high DNA integrity and purity. The best preservation method for non-live material is flash-freezing, but samples preserved in 95% ethanol or 20–25% DMSO-EDTA (for vertebrates) stored at 4 °C for up to one week also show little degradation (Dahn et al. 2022). If sampling occurs in hot climates, the use of insulated boxes, ice packs, wet ice, dry ice, or electronic coolers should be considered (Dahn et al. 2022), up to LN2-cooled dry shippers. Dahn et al. (2022) provide guidelines regarding sample preservation and choice of tissue for different vertebrates to ensure a high DNA quality. Further guidance on collection, preparation and storage of animal, plant, and fungal material to be used for WGS is provided in the PacBio technical note (2018).

RECOMMENDATION
Commercial reagents were mainly optimised for lower molecular weight DNA, and therefore, are not suitable either for uHMW preservation or for chromosomal 3D interactions (Hi-C) (Dahn et al. 2022).

Some recommendations should be followed if Hi-C methods are aimed for, as these require intact cell nuclei (Lajoie et al. 2015; ERGA 2021; **PacBio Hi-C requirements**, **National Genomics infrastructure**-Sweden):

- Tissue should be quantified: 20–200 mg soft, non-fatty animal tissue (internal organs, or muscle) is needed. Liver samples should not be used as input samples due to the amount of enzymes that can degrade DNA. Whole small animal specimens should occupy at least 50 µl. 1 ml non-nucleated blood in EDTA or heparin should be collected or 2–5 ml if flash-frozen. For nucleated blood, 200 µl flash frozen. 300 g young leaves with non-fibrous tissue.
- Cell cultures can also be used (10–50 million cells or more).
- Samples should remain frozen during transport, grinding and transfer procedures.

RECOMMENDATION
All samples that were preserved frozen with a preservation solution, in RNA later, or in ethanol, require pre-treatment before DNA extraction.

Specific DNA extraction methods are available to produce uHMW, such as bead-based methods (e.g., MagAttract HMW DNA kit), agarose plug methods (Bionano Prep Soft/Fibrous Tissue Protocol), or the the Circulomics thermoplastic magnetic disk (Nanobinds) method (Dahn et al. 2022). For HMW, the portal "**Extract DNA for PacBio**" provides a list of publications

describing extraction protocols for subsequent PacBio sequencing. Further DNA extraction protocols for third generation sequencing for several taxa can be found in the **protocols.io** repository, as well as in Green and Sambrook (2018) and Pereira (2022). Note that bead beating is required to break tough cell walls of plants, fungi, and some microorganisms, but it can also fragment DNA (Heavens et al. 2021). Li et al. (2020), Stark et al. (2020), Jones et al. (2021), and Russo et al. (2022) have optimised protocols that can overcome this disadvantage, as well as to remove contaminants (e.g., polysaccharides). For marine organisms, refer to Panova et al. (2016). For environmental samples, the **Earth Microbiome Project** (Marotz et al. 2017), Sakai (2021), and Trigodet et al. (2022) have established protocols for long-read sequencing.

If phenol-chloroform extraction protocols are used, the phenol has to be fresh and not oxidised (GTF 2020). If further purification is needed, the Qiagen Genomic-tip 500/G, MoBio PowerClean columns, or a high-salt clean-up protocol can be used for a wide range of samples (PacBio 2014; GTF 2020). Note that the latter may lead to a loss of 50% of the sample (UC Davis Genome Center 2022).

RECOMMENDATION
If possible, an RNAse digestion step should be included after DNA extraction, as samples must be RNA-free before proceeding with long-read sequencing. Further information can be found at the **UC Davis Genome Center website**.

Recording the technical value/quality of a DNA sample will allow researchers to estimate the probability of success of their planned downstream analyses. Sequencing technologies have technical requirements for successful sequencing results. If these are not met, sequencing results will not be reliable, and the sequence quality and quantity will be lower than expected.

The following table shows the ideal DNA quantities for long-read sequencing (ERGA 2021; UC Davis Genome Center 2022). Note that values can vary depending on the sample/library preparation and sequencing platform:

Table 6.

DNA	PacBio	Oxford Nanopore
Quantity	23 µg (min 15 µg) 7 µl small genomes 3.2 µl microorganisms	>5 µg
Concentration	50 ng/µl	100 ng/µl
Volume	50 µl – 400 µl	50 µl
DIN	>8	

RECOMMENDATION
The database for **long-read sequencing** provides access to existing analytical tools, and it can help in planning and performing best-practice analyses.

CHAPTER 10
DNA Assessment

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Introduction

The technical value of a DNA sample, as opposed to its biological value (i.e., taxon, collection data), can be expressed in DNA quantity and DNA quality. The latter can be measured in terms of DNA purity and DNA integrity (although some sources also subsume concentration under DNA quality). These measurements are fundamentally important to estimate the probability of success for planned downstream molecular techniques, to evaluate DNA isolation procedures, and to validate DNA extracts, which in turn, will allow for a better sample management. Note that DNA quantity, purity and integrity are influenced by several factors such as the sampling method and selected tissue (Casas-Marce et al. 2010; Nowland and Southgate 2015), the organism´s biology (Spooner and Ruess 2014), the preservation method (Nagy 2010; Wong et al. 2012; Allison et al. 2021), the DNA extraction and storage procedures (Lee et al. 2010), and the subsequent DNA sample manipulation (e.g., freeze-thaw cycles). Measurements taken right after DNA extraction will predict the quality of the sampled material and the extraction method, whereas measurements taken at a later point will assess DNA storage conditions (e.g., temperature and preservation fluid). Contamination can also affect the DNA´s technical value, and so measures should be taken to minimise the risk of contamination during sample handling, and the production of aliquots should be considered. Refer to Ruvira and Ruiz Arahal (2012) for DNA quality control validation guidelines.

RECOMMENDATION
All technical values available should be fully recorded in the collection and/or laboratory database, including gel images, used measurement methods, and for which procedure they were intended (e.g., DNA extraction, library preparation).

DNA purity

DNA purity can be assessed by measuring the absorbance using a UV spectrophotometer (e.g., NanoDrop, DeNovix, bioDrop Duo, GeneQUant Pro). Spectrophotometry methods are convenient because they can be carried out quickly, instruments are rather inexpensive and easy to use, do not require additional reagents and data analysis is relatively simple (Boesenberg-Smith et al. 2012). Ideally, Tris buffer should be preferred over water when eluting the sample to prevent inaccurate and highly variable measurements (Koetsier and Cantor 2019). It is important that the spectrophotometer is placed in a temperature-controlled area to avoid sample evaporation, due to the small sample aliquot (1–2 µl) taken for measurement (Green and Sambrook 2018).

In general, purity values are optimal when ranging from 1.8 to 2.0 at a 260/280 ratio (or 2.0 to 2.4 at a 260/230 ratio) (Lucena-Aguilar et al. 2016; Peñafiel et al. 2019; ERGA 2021; PacBio 2022), when DNA preparation have little absorbance at 320 or 230 nm, and when the spectral profile shows a smooth peak shape at 260 nm, which indicates that the DNA extraction has produced an ample nucleotide quantity (Green and Sambrook 2018). Values below 1.8 will mean that the DNA might be contaminated (e.g., with proteins, salts, ethanol, phenol) or that the sample shows a very low DNA concentration (PacBio 2022). Higher values are usually not an issue, but they may indicate that some oversight has occurred during the procedure, such as, a wrong solu-

tion having been used as negative control, the instrument not being well-calibrated, or that the pedestal being dirty (Sambrook and Russell 2001; GTF 2020). In any case, the spectral profile should be checked for abnormalities (PacBio 2022). Note that diluted nucleic acid samples may look highly contaminated, whereas concentrated ones may appear to be clean (Koetsier and Cantor 2019).

RECOMMENDATION
It is best practice to measure the absorbance at different wavelength ratios (230, 260, 280, and 320 nm) to confirm the presence of other compounds within the sample that cannot be absorbed at 260 nm, for which DNA absorbs strongly.

This method is more appropriate for pure DNA concentrations ranging between 3.5 and 90 ng/µl (Sambrook and Russell 2001; García-Alegría et al. 2020), although measurements can be unstable as slow as 20 ng/µl (Koetsier and Cantor 2019). Spectrophotometry should not be used for low concentrations, as found in small-sized organisms or impure DNA (Hodkinson et al. 2007; Wilding et al. 2009), because spectrophotometric methods are sensitive to contaminants (e.g., proteins, nucleotides, phenol) (Green and Sambrook 2018).

DNA quantification or concentration

DNA quantification is an essential calculation because specific targets are required for optimal downstream application performance (Boesenberg-Smith et al. 2012). DNA quantity is expressed as either DNA concentration (e.g., ng/µl) or DNA weight/yield (e.g., ng), which can also be assessed using spectrometry. However, it is not especially recommended because it often overestimates DNA concentrations (Green and Sambrook 2018) and it is not informative concerning degradation of DNA.

RECOMMENDATION
If there is enough DNA available, it is a good practice to take aliquots (2 µl) from the top, middle, and bottom of each DNA sample to obtain an average estimate of the DNA concentration (Dahn et al. 2022).

Another way to measure DNA quality and quantity is using agarose gels. Gels are stained, for instance, with ethidium bromide or GelRed and visualised under UV using a transilluminator, and HMW can be easily assessed with a large-range ladder, like Hind III (Mulcahy et al. 2016). DNA size bands are compared with a standard DNA ladder, and concentrations can be measured using the densitometry function of any imaging system software (Wang et al. 2017). Impurities such as detergents or probes, can usually be seen as a smear, whereas RNA impurities are often visible at the bottom of the gel, due to their faster migration. Note that classic gel electrophoresis can give inaccurate values due to dye intensity problems (Green and Sambrook 2018).

A more reliable alternative to assess concentration of nucleic acids is fluorometric determination (e.g., Qubit Fluorometer, Quantus, Tecan Genios), because it is not affected by the presence of contaminants, and because low concentrations of DNA can easily be detected (Wilding et al. 2009; Green and Sambrook 2012). It is also the preferred method for long-read sequencing. This method uses dyes (e.g., Quant-iT PicoGreen, Hoechst 33258, SYBR dyes) to stain DNA and a lambda DNA standard to help determine the DNA concentration of the samples (Green and Sambrook

2012; Wilding et al. 2009). Keep in mind that the Hoechst 33258 dye does not recognise single-stranded DNA, and hence quantitation results may be overestimated (Invitrogen-MP 2008). If working with HMW DNA, each sample should be measured in triplicate to check for reproducibility, as HMW DNA is usually not perfectly dissolved.

Spectrophotometric and fluorometric measurements should usually agree on estimated concentration values. If values are very different (≥50% difference), a bead purification step should be carried out to get a cleaner sample and hence, similar values (PacBio 2022).

For a comparison of fluorescence and absorbance methods, see the **Invitrogen technical note** (2016) or Leggate et al. (2006).

Additionally, RT-PCR and ddPCR are sensitive methods that can be performed using fluorometric probes to detect PCR inhibitors and DNA quantities, even in small amounts, and thus are also suitable for aDNA studies (Boesenberg-Smith et al. 2012; Robin et al. 2016). These amplification methods, however, require a larger investment in resources compared to other options, and DNA integrity will not be assessed in qualities needed for some NGS methods.

It is essential to pipette correctly to measure DNA concentration, especially when dealing with uHMW DNA, which is very viscous and sticky, as opposed to HMW. Koetsier and Cantor (2021) provide a shearing approach based on vortexing to produce fragments in the range of 50–100 kb, allowing easy uHMW DNA sample handling and reliable concentration measures. Furthermore, accurate pipetting is essential for DNA assessment, so in addition to proper sterilisation, pipettes should always be calibrated and should not be used at the extreme of volume specifications.

DNA integrity

DNA integrity measurements examine DNA fragmentation, meaning the length of DNA, which can be visualised using agarose gels or capillary gel instruments / DNA screen tapes (e.g., Bioanalyzer, Fragment Analyzer, Agilent TapeStation) (Forrest et al. 2019; Akinwole and Babarinde 2019). The latter can perform electrophoresis, imaging, and analysis, enabling a more automated workflow.

DNA purity can also be determined using pulsed-field gel electrophoresis (PFGE), mini-gel electrophoresis, or AFLP (Clermont et al. 2014; Green and Sambrook 2012). The PFGE (e.g., Agilent Femto, Bio-Rad CHEF Mapper XA, **Sage Science** Pippin) is a technique used to separate very large DNA molecules, and hence, it is used for long-read sequencing technologies. However, the set-up for PFGE can be expensive and time-consuming.

For a cheaper alternative, Mulcahy et al. (2016) proposed a standardised method to assess the quality and size of DNA by scoring gel images in the ImageJ software. A slow, concentrated gel is run with a large-range ladder (Hind III), and a size can be chosen for standard comparison (e.g., 23–9 kb). The size marker ~ "9 kb" (= 9,416 bp) was proposed as a standard for genomic quality in biobanking samples, and it is suitable for both short- and long-read sequencing. The detailed protocol can be found in Mulcahy et al. (2016, supplementary information). When using analytical quantification equipment, DNA degradation can be determined by calculating (from 1 to 10) the DNA integrity number (DIN). A high DIN indicates intact DNA, whereas a low DIN a very degraded sample (Agilent Genomic DNA 2015).

Note that DNA integrity can be compromised if DNA samples are not correctly handled. The following recommendations will minimise shearing and degradation (GTF 2020):

- Use wide-bore tips (~3 mm in diameter).
- Prevent or minimise vortexing.

- Pipette slowly.
- Use low-binding microfuge tubes.
- Avoid freeze-thaw cycles, overdrying and overheating.
- Samples should not contain insoluble material, denaturants, (e.g., phenol), detergents (e.g., SDS) nor RNA or carryover contamination (e.g., humid acid).
- DNA should not be exposed to high temperatures (>65°C) nor extreme pH (<6 or >9).

RECOMMENDATION

The choice of DNA assessment method will depend on the cost, time, and available equipment, as well as on the aim of the laboratory/project. In general, an inexpensive standardised method should be used for all DNA samples, except for those that demand more information for downstream analyses (e.g., candidates for NGS sequencing). If necessary, quality control can be performed by third-party companies, such as Rapid Genomics, Macrogen, Edinburgh Genomics, Genomics Core, BGI, or Imagene.

CHAPTER 11
Genomic Characterisation

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Introduction

Biodiversity banks store several types of samples and species of various groups of organisms. Nevertheless, most of them are not characterised genetically. Ideally, the genetic data of all samples should be acquired, however this is challenging to achieve. Three kinds of methods are available for genomic assessment and are explained below.

RECOMMENDATION
DNA fingerprints and DNA sequences are considered Digital Sequence Information (DSI) and ideally, they should be shared in an open database (e.g., ENA/EMBL, DDBJ, Genbank/NCBI). For further information see Scholz et al. (2020).

Whole genome sequencing (WGS)

WGS is currently considered an expensive procedure to become a general standard for genetic characterisation of biobank samples, but it has proven to be useful for identification of pathogenic bacteria (Nouws et al. 2020; Pan et al. 2020; Hahm et al. 2022) and viruses (Charre et al. 2020). The biggest constraints on *de novo* sequencing are genome size and repetitive sequences that will produce irrelevant data for identification purposes, an obstacle most relevant in plants (Hollingsworth et al. 2016). Detailed guidance for engaging in WGS can be found in Ekblom and Wolf (2014). An example of genomic characterisation of vertebrates is described in Keinath et al. (2015) and of plants in van Buren et al. (2015).

On the other hand, complete organellar genomes (plastid or mitochondrial) are increasingly used to identify species. They can be obtained by genome skimming, target enrichment or direct isolation protocols and enable further discrimination among taxa (Hollingsworth et al. 2016; Tonti-Filippini et al. 2017). Note that even complete plastid genome sequences may not distinguish lower taxonomic levels (e.g., subspecies) and hybridisation events may hinder taxon identification (Tonti-Filippini et al. 2017; López et al. 2021). Refer to Yang et al. (2013) for some examples in plants, and to Franco-Sierra and Díaz-Nieto (2020) for examples in animals.

Single Nucleotide Polymorphism (SNP) Arrays

SNP arrays have been developed both for single and multi-species approaches to assess genetic diversity. Biobanks may specifically benefit from the latter by being able to assess several species at once. Ideally, arrays should capture the variety of breeds/populations within species on all chromosomes, as well as the variation of SNPs used for ancestry, mitochondrial SNPs, MHCs, and QTL regions. SNP arrays can also be used for sex determination, kinship testing, and mtDNA haplotyping and the results can be compared to test for genetic differences that occur over time (IMAGE 2020).

SNP arrays have been developed for economically important groups such as oysters (Gutierrez et al. 2017), fish (Palti et al. 2015; Nugent et al. 2019; Mastrochirico-Filho et al. 2021; Peñaloza et al. 2021), chicken (Kranis et al. 2013),

various other livestock (IMAGE 2020), crops (You et al. 2018; Montanari et al. 2019; Keeble-Gagnère et al. 2021), and hardwood species (Silva-Junior et al. 2015; Bernhardsson et al. 2021; B4EST Project 2021). In addition, some commercial chips have been successfully applied to non-model species (Pertoldi et al. 2010; Haynes and Latch 2012; Minias et al. 2019), whereas very few chips have been developed exclusively for specific wildlife species (Van Bers et al. 2012; Kawakami et al. 2014; Humble et al. 2020) and for plant pathogens (Capote et al. 2012).

DNA barcoding

DNA barcoding is a fast and accurate method for identifying and characterising species, especially cryptic or unknown, and for assessing biodiversity (Moura et al. 2018). The ideal barcode is a standardised universal short DNA sequence (400–800 bp) that remains stable through many generations within a species, but at the same time, is variable enough to allow discrimination between closely related species (Kress and Erickson 2012a; Hollingsworth et al. 2016). In some taxa, however, DNA barcoding has specific limitations due to e.g., the existence of pseudogenes, heteroplasmy, and hybridisation, as well as slow mutation rates or rapid divergence (Hollingsworth et al. 2016; Gong et al. 2018). While adding a DNA barcode to a sample is a most expedient approach, it needs to be emphasised that a reference database relying on a priori species identification by morphological or other (e.g., behavioural) traits is crucial.

Comprehensive guidelines for DNA barcoding, including metadata standards for specimen and DNA curation, have been developed by Rimet et al. (2021) and the Centre for Biodiversity Genomics, University of Guelph (2021).

Protists and algae

For protist diversity, several barcodes from plastids, the mitochondria, and the nuclear DNA are used (Pawlowski et al. 2012; Warren et al. 2017). The variable V4 region of the nuclear SSU (ribosomal small subunit) rRNA gene is the most widely used barcode marker in both terrestrial and marine protists (e.g., flagellates, amoeboid protists, foraminifera, diatoms) (Amaral-Zettler et al. 2009; (Kopf et al. 2015; de Groot et al. 2016; Burki et al. 2021). Still, the design of a universal DNA barcode is difficult because of the independent and complex evolutionary histories of protists (De Luca et al. 2021). It must be noted that co-amplification of non-target DNA can occur without distinction in barcode markers (e.g., V4 and V9 regions of the nrSSU rRNA gene–ribosomal small subunit–rRNA) possessing several copies due to polyploidy, gene duplication events, or the presence of two nuclei (in ciliates) (Altermatt et al. 2015; Geisen et al. 2018). For myxomycetes, the nrSSU rRNA and the EF-1a (elongation factor-1a) genes are the most widely used markers for molecular genetic analyses (Schnittler et al. 2017; Walker et al. 2017; Wang et al. 2021).

Identification of microalgae and cyanobacteria is carried out using the nrSSU rRNA gene, the variable domains D2/D3 of the nrLSU (large ribosomal subunit) rRNA gene, the plastid-encoded *rbc*L (*ribulose 1,5-biphosphate carboxylase large subunit*) gene, the nuclear-encoded ITS–2 (Internal Transcribed Spacer within the rRNA gene cluster) region, and the plastid-encoded psbA/*rbc*L spacer (Hadi et al. 2016; Friedl and Lorenz 2012). For diatoms, the most suitable DNA barcode markers are the 5.8S rRNA gene + ITS–2 barcode (Moniz and Kaczmarska 2009) or the *rbc*L-3P gene (Hamsher et al. 2011).

Several DNA barcoding loci, such as nrLSU-D2/D3, COX-1, COX-2, COX-3 (mitochondrial cytochrome c oxidase subunit 1, subunit 2 and subunit 3 genes) and UPA (domain V of the plastid 23S rRNA gene known as the "univer-

sal plastid amplicon") have been employed for the genetic characterisation of red and brown macroalgae, although in general COX-1, *rbcL*, and *rbc*S (small subunit) are the preferred loci for these algae (Leliaert et al. 2014; Bartolo et al. 2020). It is more problematic, however, to develop a COX-1 barcode for green algae, due to the presence of introns within this region (Saunders and Kucera 2010). The marker genes *rbc*L and *rbc*S are especially suitable as DNA barcode sequences for old specimens, since their barcode amplicons have a small size (Bartolo et al. 2020). Studies on green algae have usually relied on the *rbc*L, nrSSU rRNA, and nrITS rRNA loci. A promising additional candidate to become a standard marker for green algae, excluding Cladophorales, is the *tuf*A (plastid elongation factor) gene (Saunders and Kucera 2010; Leliaert et al. 2014). In general, there is increasing evidence that the application of multiple markers, preferably from different genomic compartments (nuclear, plastid and mitochondrial) increases the effectiveness of species identification (e.g., Leliaert et al. 2014).

Note that currently it is not possible to assign some protist DNA sequences unambiguously to a genus or species, due to the occurrence of incorrectly labelled sequences, lack of classified sequences in the databases, or simply because no reference sequences have been deposited yet (e.g., when the species is new to science) (Mäki et al. 2017; Geisen et al. 2018; Gooday et al. 2020).

Fungi

Molecular identification of fungi is usually achieved by sequencing the nuclear ITS barcode locus (Schoch et al. 2012; Kõljalg et al. 2013; Ryan et al. 2019). The ITS locus has also been successfully applied to lichens both in archival and fresh material (Kelly et al. 2011; Dal Forno et al. 2022). However, it is sometimes necessary to use secondary barcodes to distinguish species more accurately (Stielow et al. 2015; Lücking et al. 2020). These markers include the large and the small subunits of the nuclear ribosomes (nrLSU, nrSSU), the small subunit of mitochondrial ribosomes (mtSSU) (especially for lichens), the *tef*1–α gene (translation elongation factor 1-α), and the *rpb*–1 and *rpb*–2 genes (DNA-directed RNA polymerase II subunit 1 and subunit 2) (Schoch et al. 2012; Kistenich et al. 2019; Tekpinar and Kalmer 2019). For DNA-based determination of arbuscular mycorrhizal fungi follow Kolaříková et al. (2021).

Lücking et al. (2020) made four essential recommendations to improve fungal DNA-based identification, which equally hold true for barcoding approaches in other taxa:

- Variation within and among barcode markers should be recorded.
- Sequences from missing and underrepresented taxa should be included in data repositories.
- Sequences should be properly curated.
- Sequences should be linked to a specimen voucher.

Plants

Due to their lower resolving power in tracking species boundaries, no single plastid marker, or any of the rRNA genes can be compared with the near-universality of COX-1 as a DNA barcode gene in animals (Hollingsworth et al. 2016; Hua et al. 2022). However, the combination of barcode markers, such as the protein-encoded loci *rbcL* and matK (*maturase K*), found in the plastid genome, has provided a good basis for species identification (CBOL Working Group et al. 2009; Vasconcelos et al. 2021). In addition, the ITS region of the nuclear rRNA genes and the plastid non-coding spacer *trnH-psbA* have been used as secondary DNA barcodes (Kang et al. 2017). The plastid *trnL* intron-P6 loop has also been used for degraded and environmental plant DNA (Hollingsworth et al. 2016). Hua et al. (2022) developed a DNA signature sequence (i.e., sequences used to distinguish one taxon from all other taxa) approach that seems to be promising for identifying and validating plant species.

Animals

The COX-1 region has been widely and successfully used for most animal taxa, except a few exceptions, e.g., Cnidaria, for which the mitochondrial rRNA gene for the small ribosomal subunit (16S) was established as a more suitable marker (Herbert et al. 2003; Moura et al. 2018). Further details and protocols on animal DNA barcoding can be found in Kress and Erickson (2012b). Metazoan-level universal single-copy orthologs (metazoan USCOs), a set of nuclear-encoded protein-coding genes, are universal markers suited for species delimitation that outperform mitochondrial DNA barcodes (Eberle et al. 2020; Dietz et al. 2022). Note that reference genomes are required for bait designing to sequence USCOs, which may be challenging for organisms difficult to sample (Dietz et al. 2022).

Note: In most genetic resource centres, molecular markers are used as a tool for improving the management of ex situ collections. Traditionally, microsatellites, RAPD, AFLP or RFLP fingerprints and ITS profiles have been used to assess the genetic diversity and stability before and after storage procedures and to confirm genetic identity (Capote et al. 2012; Homolka 2014; Luchi et al. 2020). AFLP has been the most commonly used method in bacteria, animals, plants, and microalgae, since it is sensitive to DNA methylation and allows the analysis of many loci scattered along the whole genome, without having prior sequence knowledge of the examined organism (Müller et al. 2007; Friedl and Lorenz 2012). Genetic characterisation can also help to identify duplicates and changes between explants in the field, stored in vitro, and/or cryopreserved (Reed et al. 2004; Sarasan et al. 2006; Martín et al. 2013).
Newer techniques based on genome-wide sequencing show a higher resolution and better reproducibility (Mascher et al. 2019). Markers like SNPs (Single Nucleotide Polymorphism), DArTs (Diversity Array Technology), ISSR (Inter Simple Sequence Repeat), IRAP (inter-retrotransposon amplified polymorphism), and EST-SSR (expressed sequence tag-simple sequence repeat) are currently used (Koebner 2003), as well as the MSAP (methylation-sensitive amplified polymorphism) to determine epigenetic changes (Matsumoto 2017). In addition, flow cytometry can be a complementary procedure to identify changes in ploidy levels and DNA content (Paunescu 2009). Karyotyping is also a standard protocol for the authentication of cell lines, and ideally, cell lines should be accompanied by an STR certificate to assure their authenticity (Goswani et al. 2022).
Microbial diversity in environmental samples and their possible temporal structural changes can be assessed by using either the automated ribosomal intergenic spacer analysis (ARISA) fingerprinting method, or metagenomics (van Dorst et al. 2014; Parmar et al. 2019; Sraphet and Javadi 2022). Note that it may be difficult to describe fingerprints from unculturable microorganisms.

Note: a very crucial step is to associate genetic data with morphological information of different life stages, images, and the exact taxon name. Online fungal repositories for new species names/descriptions and identification should always be checked: **Index Fungorum**, **MycoBank** and **Fungal Names**, **U.S. National Fungus Collections Databases** (Smith et al. 2020; Crous et al. 2021). Documentation is also available for **marine fungi** records.
For protists two databases are available: the **Tara Oceans database**, and the Protist Ribosomal Reference database PR2 (Schoenle et al. 2021). Further, the WFCC has developed the **MIRCEN World Data Centre for Microorganisms**, which is a comprehensive microbial resource (Wu et al. 2017). The StrainInfo bioportal has also been developed to increase discoverability of microorganism resources (Dawyndt 2002). Another reference resource is the

SILVA rRNA database, which provides datasets for bacteria, archaea, and eukaryotes (Quast et al. 2013). The **WoRMS** database also provides information regarding classification and catalogue of marine and non-marine taxa. Refer to Sun et al. (2015) for a complete list of web resources of microbial data.

For plants, scientific names can be checked in the **Tropicos** and the **Catalogue of Life**. Plant names can be compared with type material using the **Jstor** database (Trujillo-Argueta et al. 2021). **COPO** is a portal for plant scientists designed to store and retrieve omics data. Scientific names of animals can also be checked in the **Catalogue of Life**.

BOLD (Barcode of Life Data System) is a general open access database that facilitates the acquisition, storage, analysis, and publication of DNA barcode records (Gong et al. 2018). It functions as a reference library for known and unknown species, as it also links metadata and images with DNA sequences. Further information can be found in Ratnasingham & Hebert (2007).

CHAPTER 12
Ethics

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Introduction

Biodiversity and environmental biobanks are responsible not only for the conservation and management of biological samples but also for the correct, transparent, and trackable process of samples and associated data throughout the biobanking workflow (i.e., collection, preservation, storage, utilisation, distribution/supply, and possible destructive sampling, data generated and publications). To accomplish the latter task, biobanks should comply with legal standards and international regulations, such as the ITPGRFA, CITES, and CBD, including the NP, when ratified (D´Amato et al. 2020).

The CBD provides framework legislation through national laws to support the conservation of biodiversity, the sustainable use of biodiversity and the fair and equitable sharing of benefits arising from that use. The NP is a legally enforceable mechanism to ensure access and benefit sharing arising from the utilisation of genetic resources in a fair and equitable way. William and Wolfson (2006) and Applequist (2014) provide further information on the legal issues in DNA banking, and information on Rights Management and Access and Benefit Sharing can be found in de Mestier et al. (2022). The transparent user-friendly system of transfer (Belspo 2016) and the "MicroOrganisms Sustainable use and Access regulation International Code of Conduct (MOSAICC)" have been developed to support CBD implementation at the microbial level (Becker et al. 2019). CITES aims to protect endangered species from the threats of international trade and involves regulations on the import/export of fauna and flora. ITPGRFA facilitates access to the genetic materials of 64 crops in a multilateral system for research, breeding and training for food and agriculture. Those accessing genetic materials through the system agree to share benefits through mechanisms established by the treaty (FAO 2014). Policies and the impact of international regulations on animal genetic resources are explained in Hiemstra et al. (2006), Blackburn et al. (2014), Comizzoli and Volt (2016), and Martyniuk et al. (2018).

Additionally, biobank staff and researchers should be aware of ethical issues associated with collections that arise from various other situations:

Illegal samples

All samples that are deposited in a biobank must be legally collected. Biobanks are also obliged to guarantee that samples donated by third parties are accompanied by all necessary legal paperwork (Applequist 2014; Ståhls et al. 2021; Sherkow et al. 2022). Illegal samples should be rejected (Prendini et al. 2002). Note that certain samples from zoos, museums and botanical gardens may have vague information regarding their origin, so researchers should be attentive of any ethical violation and constraints of their uses (Sherkow et al. 2022), and apply careful due diligence procedures before use.

Sample collection and engagement of local communities

During fieldwork, environmental damage and disturbance/stress to non-targeted organisms should be minimised (Bennet 1999; Gales et al. 2009). Moreover, researchers should avoid the unintended transport of invasive species or pathogens when sampling at various locations (Costello et al. 2016). Rare and threatened species must not be collected because this may contribute to their decline, unless there is a valid explanation and explicit permission to do so (Costello et al. 2016; Alba and Islam 2019). Oversampling and large-scale collecting methods (e.g., fogging, netting) that kill non-target organisms should be also avoided (Costello et al. 2016; Sherkow et al. 2022). Keep in mind that sensitive data or specimens must be treated carefully to prevent illicit hunting/collection or import of endangered species. Clemann et al. (2014) and the Animal Research Review Panel (2020) provide guidelines for ethical vertebrate

voucher collection. Costello et al. (2016) provide a list of considerations for respectful conduct during biological field sampling and Ramírez-Castañeda (2022) provides a set of principles for equitable fieldwork in biology.

Institutional ethics committees should require that all specimens collected in the field are deposited with their respective data in an accredited research collection or formalised biobank for their long-term preservation and availability (Groeneveld et al. 2016; McLean et al. 2016; Sikes et al. 2016). Although it is common practice for animals and plants (Culley 2013; Clemann et al. 2014) it remains rare for microorganisms (Becker et al. 2019).

Permits and permissions for fieldwork (e.g., collection, genetic resources access, CITES) on public or private lands, whether domestic or foreign must be obtained for all types of samples, including palaeontological samples, before collecting (SVP 2021). Moreover, national laws covering Access and Benefit Sharing (ABS), including prior informed consent, and Material Transfer Agreements (MTA) must be fully followed (Ramírez-Castañeda 2022). Note that extra collection permits, historic preservation permits, or community letters of support may be required if fieldwork is carried out on territories that belong to indigenous peoples and local communities (Posth et al. 2018; Wagner et al. 2020; Sherkow et al. 2022).

Indigenous peoples, local communities and farmers have the right to preserve their territories, their traditional knowledge and farming practices, and hence, have a claim to certain species/landraces/breeds (UN 2006; Engels et al. 2011; Sherkow et al. 2022). Communities should understand social implications of the research studies, and researchers should engage communities in their activities by implementing the following ethical principles (ISE 2006; Engels et al. 2011; Wagner et al. 2020):

- Obtain prior informed consent by formally consulting with the communities to explain the research process and analyses, as well as data ownership and data distribution. Thus, cultural, and intellectual heritage rights will not be violated. Communication is essential before, during and after research, especially when working on human ancient DNA.
- Respect tribal sovereignty, understand relevant beliefs and community preferences and preserve their role as guardians of their territories.
- Active participation throughout the entire research procedure, along with full disclosure of the research methodology, plans for developing long-term responsibility, reporting/managing data, and stewardship of the collections.
- Active protection to maintain the cultural and biological diversity.
- Take precautionary measures of identifying, assessing and preventing potential cultural and biological damage due to research outcomes. In case this occurs, appropriate restitution should be considered together with the community.
- Support indigenous research and capacity building.
- Respect indigenous "inalienability", i.e., the right to be unaffected by the introduction of new foreign knowledge or inventions.
- Respect indigenous confidentiality and the right to exclude information from publications concerning their culture, traditions, and beliefs.
- Acknowledge and apply all due credit for their contributions to research.

If appropriate, local people and farmers can be involved in the decision-making to choose which species/breeds/individuals should be prioritised for biobanking (IMAGE 2020). In addition, associated knowledge from the communities can also be collected (especially when sampling and preserving genetic resources from agricultural species), always recognising property rights (Engels et al. 2011). The Care Principles for indigenous data governance outline considerations concerning the use of data in which indigenous peoples may have rights and interests (Gida global 2018). Further details regarding agricultural biodiversity ethical issues can be found in Canal-Forgues (1993), SGRP (2003) and Engels et al. (2011). Refer to Wagner et al. (2020), Alpaslan-Roodenberg et

al. (2021), and Tsosie et al. (2021) for further ethical practices and processes in palaeogenomics and DNA research.

Animal welfare

Several ethical guidelines (e.g., Nuffield Council on Bioethics 2005; Gales et al. 2009; Sikes et al. 2016) have been developed for the treatment of animals both in the field and in the laboratory and comprise the following criteria:

- Capturing. Attempts to capture an animal should be limited, as chasing can be stressful for the animals (Bennet 1999). Once captured, the enclosure time should be minimised, animals should be monitored (e.g., temperature, physical trauma) and fed, if necessary. Note that excessive handling can lead to the animal´s death (Bennet 1999).
- Tissue biopsy sampling. Potential infections should be minimised, and any wounds produced should be small and clean so that they can heal easily. Specific surgical procedures or projectile biopsy sampling should be performed by licensed, trained, and experienced researchers. Hunted and stranded animals, along with bycatches, should be considered as a source of tissue sample for genetic studies. Furthermore, non-invasive techniques should be preferred over invasive sampling to minimise stress and pain in animals (Zemanova 2019; D´Amato et al. 2020).
- Release of captive animals in good condition and in the right place.
- Euthanasia. Lethal methods should be avoided as much as possible (Costello et al. 2016; Zemanova 2019) but, if necessary, they should be painless and performed swiftly (Sherkow et al. 2022). Ideally, a veterinarian should provide guidance to the researcher and the method of euthanasia has to be approved by the institutional animal care and use committee. Note that the selected method needs to be appropriate for the species and for the animal´s age (Nebraska University 2019; D´Amato et al. 2020).

Archaeological and human remains

Human remains are not cultural objects (Ragsdale 2012). They are often described as specimens rather than people, so a more respectful terminology should be used (Wagner et al. 2020). Furthermore, some ethical challenges can arise when research questions the existence or identity of culturally related descendants (Austin et al. 2019). Storage of human remains should suit the beliefs and traditions of the community provenance (National Museum of Ireland 2019), unless repatriation processes are requested by indigenous peoples after research conclusion (Wagner et al. 2020). Collections should adhere to all relevant guidelines for the care of human remains, such as the International Council of Museums code of ethics (ICOM 2017), the guidelines for care of human remains in museums and collections (Deutscher Museumsbund 2021), or the Human Remains Policy from the National Museum of Ireland (2019). Additionally, Austin et al. (2019) suggested a set of best practices for the appropriate curation of anthropological collections to be used for molecular studies.

Archaeofaunal and palaeoethnobotanical remains, on the other hand, are connected to human activities. The preservation of these types of remains is included in most ethical guidelines in archaeology. Unfortunately, these samples are often neglected, inaccessible and commonly discarded mainly due to museum financial constraints (Salick et al. 2014; Pálsdóttir et al. 2019). The international council for archaeozoology summarises standards and best practices in archaeozoology (ICAZ 2009), and Pálsdóttir et al. (2019) provide ethical guidelines for collection and destructive sampling of archaeofaunal remains. Salick et al. (2014) offer standards for curating and preserving palaeoethnobotanical and archaeofaunal samples, as well as the legal aspects of such cultural collections.

Sample disposal

Deaccession or sample disposal has to be taken into account and occurs in cases when for example storage constraints, redundancy

of duplicate specimens/cultures, or compromised specimen/DNA integrity apply, or when a sample is rendered useless due to data loss or compromised authenticity (DMNS 2017; ISBER 2018). Policy and procedure guidelines for deaccessioning should be followed (e.g., American Association of Museums or **Museums Association** UK) and, ideally, the curator/manager should carry this out either by destroying the samples or by transferring them to another institution or by repurposing them for educational use (DMNS 2017; ISBER 2018; SVP 2021). Deaccessioning should be tracked and recorded in the collections management data system, stating the reasons that led to this decision (ISBER 2018). Applequist (2014) suggested including proper disposal issues in ethics training when possible.

Although out of the current biobank scope, some ethical issues appear when using cryobanked material for cloning, genetic rescue, or de-extinction purposes (Novak 2018; Holt and Comizzoli 2021), leading to unresolved moral hazard issues that will need to be discussed at all levels in the near future (IUCN SSC 2016). Sandler et al. (2021) provide frameworks to help determine when these procedures are ethically justified.

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Preface

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Chapter 3: Field Collection

Data collection

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Chapter 4: Culture Preservation and Storage Methods

Protists and fungi

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Chapter 6: Freeze-Drying or Lyophilisation

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Chapter 8: DNA

DNA extraction and ancient DNA

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Chapter 12: Ethics

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ANNEX I

Biodiversity and environmental biobanks and biobank networks

The following list comprises only those biobanks and networks that were encountered while compiling this handbook - it is not exhaustive and intended as a starting point for additional research. Further DNA banks and genetic resource repositories in the United States can be found in **iDigBio** (Integrated Digitized Biocollections).

Table A1. Protist and fungal culture collections.

Belgian Coordinated Collections of Microorganisms	BCCM	Belgium
Culture Collection of Autotrophic Organisms	CCALA	Czech Republic
Culture Collection of Algae	CAUP	Czech Republic
Institut Pasteur	IP	France
Centre International de Ressources Microbiennes	CIRM	France
Roscoff Culture Collection	RCC	France
German Collection of Microorganisms and Cell Cultures	DSMZ	Germany
Culture Collection of Algae at the University of Duisburg-Essen	CCAC	Germany
Culture Collection of Algae at Göttingen University	SAG	Germany
Culture Collection of Cryophilic Algae	CCCRYO	Germany
Benaki Phytopathological Institute	BPI	Greece
Mycotheca Universitatis Taurinensis	MUT	Italy
Microbial Culture Collection	ITEM	Italy
Microbial Strain Collection of Latvia	MSCL	Latvia
Westerdijk Fungal Biodiversity Institute (former Centraalbureau voor Schimmelcultures)	CBS	Netherlands
Polish Collection of Microorganisms	PCM	Poland
Micoteca da Universidade do Minho	MUM	Portugal
Coimbra Culture Collection of Algae	ACOI	Portugal
Russian Collection of Microorganisms	VKM	Russia
Spanish Type Culture Collection	CECT	Spain
Spanish Bank of Algae	BEA	Spain
Basque Microalgae Culture Collection	BMCC	Spain
Bioscience Genetic Resources Collection	CABI	UK
United Kingdom National Culture Collection	UKNCC	UK
European Collection of Cell Cultures	ECACC/HPACC	UK
Culture Collection of Algae and Protozoa	SAMS-CCAP	UK
Collection of Fungal Pathogens and Symbionts of Insects	CEPAVE	Argentina
Coleção de Culturas Micoteca UM, Universidade Federal de Pernambuco	UFPE	Brazil
Fundación Oswaldo Cruz	FIOCRUZ	Brazil

Culture Collection of Freshwater Microalgae from the Federal University of Sao Carlos	CCMA-UFSCar	Brazil
Canadian Collection of Fungal Cultures	CCFC	Canada
Microbial Collection, Pontificia Universidad Javeriana	CMPUJ	Colombia
Cereal Disease Laboratory	USDA-ARS	USA
Collection of Zoosporic Eufungi	CZEUM	USA
The fungal Genetics Stock Centre	FGSC	USA
Provasoli-Guillard National Center for Culture of Marine Phytoplankton	NCMA, former CCMP	USA
Culture Collection of Algae at the University of Texas at Austin	UTEX	USA
Penn State Microbiome Center		USA
Research Center of Genetic Resources, National Agriculture and Food Research Organization	NARO	Japan
Philippine National Collection of Microorganisms	PNCM	Philippines
CSIRO Algae Culture Collection	ANACC	Australia
Australian Plant Pathology and Mycology Herbarium	DAR	Australia
Queensland Plant Pathology Herbarium	BRIP	Australia
International Collection of Microorganisms from Plants	ICMP	New Zealand

Further culture collections can be found via the WFCC or DSMZ.

Table A2. Animal tissue, cell cultures, DNA collections and veterinary biobanks.

Zoo Antwerp		Belgium
Copenhagen Zoo		Denmark
German Cell Bank for Wildlife „Alfred Brehm"		Germany
Leibniz Institute for Zoo and Wildlife research	IZW	Germany
Leibniz Institute for the Analysis of Biodiversity Change	LIB/ZFMK	Germany
Reserve de la Haute Touche		France
National Institute for Ocean Science	IFREMER	France
The Mediterranean Marine Mammals Tissue Bank		Italy
Italian Biobank of Veterinary Resources	IBVR	Italy
Istituto Zooprofilattico Sperimentale delle Venezie biobank	IZSVe	Italy
Royal Netherlands Institute for Sea Research	NIOZ	Netherlands
Centre of Marine Sciences	CCMAR	Portugal
Threatened Wildlife Cell Bank		Spain
Toralla Marine Science Station of University of Vigo	ECIMAT	Spain
British National Fish Tissue Archive		UK
Nature's SAFE		UK
RZSS WildGenes Biobank		UK
Biobank Merseyside		UK
Biobank at Buenos Aires Zoo		Argentina
Marine Mammals Specimen Bank	BAMM	Brazil
Canadian Wildlife Service Specimen Bank		Canada

World Fisheries Trust		Canada
Toronto Zoo Biobank		Canada
Alexander von Humboldt Institute, Tissue Collection	IAvH-CT	Colombia
Alaska Frozen Tissue Collection		USA
Frozen Zoo at the San Diego Zoo		USA
SeaWorld & Busch Gardens		USA
Ocean Genome Legacy Centre		USA
Revive & Restore		USA
Cornell Veterinary Biobank		USA
South African National Biodiversity Institute	SANBI	South Africa
Ukutula Conservation Center & Biobank	UCC	South Africa
Dubai Aquarium and Underwater Zoo		Dubai
Kunming Wild Animal Cell Bank		China
Center for Cellular and Molecular Biology	LaCONES	India
National Bureau of Fish Genetic Resources	ICAR- NBFGR	India
Iranian Biological Resource Centre	IBRC	Iran
The National Institute for Environmental Studies	NIES	Japan
Japanese Collection of Research Bioresources Cell Bank	JCRB	Japan
Riken BioResource Research Center	RIKEN	Japan
Library of Marine Samples	KIOST	South Korea
Conservation Genome Resource Bank for Korean Wildlife	CGRB	South Korea
Genome Resource Bank- Zoological Park		Thailand
CellBank		Australia
Australian Frozen Zoo		Australia
Ian Potter Australian Wildlife Biobank		Australia
Taronga Conservation Society		Australia
CSIRO National Research Collections	NRCA	Australia

Table A3. Museums / natural history collections and botanical gardens with repositories.

Meise Botanic Garden	Meise BG	Belgium
Natural History Museum of Denmark		Denmark
Leibniz Institute for the Analysis of Biodiversity Change (Biobank at Museum Koenig)	LIB/ZFMK	Germany
Botanic Garden and Botanical Museum Berlin-Dahlem	BGBM	Germany
Finnish Museum of Natural History Luomus		Finland
Muséum National d'Histoire Naturelle	MNHN	France
National Museum of Ireland, Irish Cetacean Genetic Tissue Bank		Ireland
Perennial Plant Germplasm Repository	PPGR	Italy
Natural History Museum of London		UK
Instituto de Pesquisas Jardim Botânico do Rio de Janeiro		Brazil
Smithsonian Institution Biorepository	SI	USA
Cincinnati Zoo and Botanical Garden		USA

The Museum of Vertebrate Zoology at Berkeley	MVZ	USA
Museum of Comparative Zoology Harvard	MCZ	USA
The American Museum of Natural History, Ambrose Monell Cryo Collection	AMNH/AMCC	USA
Pacific Center for Molecular Biodiversity	PCMB	USA
Denver Botanic Gardens		USA
Missouri Botanical Garden		USA
New York Botanical Garden		USA
North Carolina Arboretum Germplasm Repository	TNCAGR	USA
National Zoological Gardens	NZG	South Africa
Steinhardt Museum of Natural History		Israel
Australian Plant DNA Bank		Australia
Australian Museum	ABTC	Australia

Many (but not all) members of GGBN represent the museums and botanic gardens community. A complete list of marine biological collections can be found in Collins et al. (2021).

Table A4. Livestock germplasm collections.

Friedrich-Loeffler-Institut	FLI	Germany
National Institute for Agricultural Research	INRA	France
French Nationale Cryobank		France
Centre for Genetic Resources of Wageningen University	CGN	Netherlands
NordGen		Norway
Banco Nacional de Germoplasma Animal	BNGA	Spain
Agricultural Research and Education Centre Raumberg-Gumpenstein		Austria
National Agricultural and Food Centre	NPPC	Slovakia
Norwegian Genetic Resources Centre	NIBIO	Norway
Cryobanque Wallonne		Belgium
Animal Germoplasm Cryobank "Giuseppe Rognoni"	IBBA-CNR	Italy
Department for Environment Food & Rural Affairs	DEFRA	UK
USDA National Animal Germplasm Program	USDA-ARS	USA
Centre for Tropical Livestock Genetics and Health	CTLGH	Ethiopia
International Livestock Research Institute	ILRI	Kenya
Research Center of Genetic Resources, National Agriculture and Food Research Organization	NARO	Japan
Animal Population Culture Collection	APCCC	China

Please refer to Eugena for more biobanks in Europe.

Table A5. Parasites.

Instituto de Investigaciones Marinas	Spain
Manter Lab of Parasitology	USA

Table A6. Seed banks, clonal plant collections, and forest genetic resources.

Seed Bank at Meise Botanic Garden	MEISE BG	Belgium
Research Institute for Crop Production	CRI	Czech Republic
Leibniz-Institute for Plant Genetic and Crop Research	IPK	Germany
National Collection of Forest Genetic Resources	CRGF	France
National Plant genomic Resources Centre	CNRGV	France
Svalbard Global Seed Vault		Norway
Kostrzyca Forest Gene Bank		Poland
Millennium Seed Bank Project Kew	MSBP	UK
Agricultural Research Corporation	EMBRAPA	Brazil
Alliance of Biodiversity International	CIAT	Colombia
Alexander von Humboldt Institute, Seed Collection	IAVH-CS	Colombia
SAGARPA National Centre of Genetic Resources	CNRG	Mexico
International Potato Centre	CIP	USA
United States Department for Agriculture, Seed Lab	USDA-NSL	USA
USDA National Plant Germplasm System	USDA-NPGS	USA
San Diego Zoo Native Plant Seed Bank		USA
Ethiopian Biodiversity Institute	EBI	Ethiopia
Plant Genetic Resources Research Institute	PGRRI	Ghana
Genetic Resources Research Institute	GeRRI	Kenya
International Institute of Tropical Agriculture	IITA	Nigeria
South African National Biodiversity Institute	SANBI	South Africa
National Plant Genetic Resources Centre	NPGRC	Zambia
National Bureau of Plant Genetic Resources	NBPGR	India
Research Centre of Genetic Resources, National Agriculture and Food Research Organization	NARO	Japan
Australian Grains Genebank		Australia
Australian PlantBank		Australia
CSIRO Seed Centre	ATSC	Australia

Further crop biobanks can be found in WIEWS (World Information and Early Warning System on Plant Genetic Resources for Food and Agriculture) and CGIAR (Consultative Group on International Agricultural Research).

Table A7. Environmental specimen banks.

Faroe Island Environmental Specimen Bank		Denmark
Tissue and Data Bank for Greenland, National Environmental Research Institute		Denmark
Finnish Environmental Specimen Bank		Finland
ANDRA Observatoire Perenne de l'Environnement	OPE	France
Mytilothèque		France
German Environmental Specimen Bank	UPB at UBA	Germany
Antarctic Environmental Specimen Bank	BCAA	Italy
Norwegian Environmental Specimen Bank		Norway

Norwegian Institute for Water Research	NIVA	Norway
Biscay Bay Environmental Biospecimen Bank	BBEBB	Spain
Environmental Specimen Bank of Galicia	ESBG	Spain
Swedish Environmental Specimen Bank	ESB	Sweden
Predatory Bird Monitoring Scheme	PBMS	UK
Canada's National Aquatic Biological Specimen Bank		Canada
Biospecimen Science Group at National Institute of Standards and Technology	NIST	USA
Yangtze Environmental Specimen Bank, Tongji University		China
Shanghai Academy of Environmental Sciences		China
Polar Research Institute of China		China
Japanese Environmental Specimen Bank for Global Monitoring	es-BANK	Japan
Japanese Environmental Specimen Time Capsule Program	NIES	Japan
National Environmental Specimen Bank	NESB	South Korea
Australian Environmental Specimen Bank		Australia

Refer to Chaplow et al. (2021) for further details on environmental specimen banks.

Table A8. Human remains / anthropological tissue banks.

Institute of Pre- and Protohistory and Medieval Archaeology, Department of Early Prehistory and Quaternary Ecology, Eberhard Karls University of Tübingen	Germany
Bog Bodies Tissue Sample Bank, National Museum of Ireland	Ireland
The international ancient Egyptian mummy tissue bank, University of Manchester	UK
University College London	UK

Table A9. Network initiatives.

General	European, Middle Eastern and African Society for Biopreservation and Biobanking	ESBB
	Global Genome Biodiversity Network	GGBN
	National Biodiversity Network	NGN
Microorganisms	UNESCO Microbial Resources Centres Network	MIRCEN
	European Culture Collection Organisation	ECCO
	European Consortium of Microbial Resources Centres	EMBaRC
	Global BRC Network	GBRCN
	Microbial Resource Research Infrastructure	Mirri
	Federación Latinoamericana de Colecciones de Cultivos	FELACC
	Colecciones Microbiológicas de la Red Paranaense	CMRP
	US Culture Collection Network	USCCN
	US National Plant Diagnostic Network	NPDN
Plants	Millennium Seed Bank Partnership	MSBP
	Mid-Atlantic Regional Seed Bank	MARSB
	Australian Seed Bank Partnership	ASBP
	Consultative Group on International Agricultural Research	CGIAR

Aquaculture and Livestock	Aquaculture Infrastructures for Excellence in European Fish Research	AquaExcel
	European Genebank Network for Animal Genetic Resources	EUGENA
	Brazilian Agricultural Research Corporation	EMBRAPA
Wildlife	FrozenArk	
	CryoArks	
	Pan-Smithsonian Cryo-Initiative	
	Biodiversity Collections Network	BCON
	FAUNA Research Alliance	FAUNABank
	Amphibia Bank	
	Biodiversity Biobanks South Africa	SAS
	EAZA Biobanking	
Marine biodiversity	European Marine Biological Research Infrastructure Cluster	EMBRIC
	Marine Biodiversity and Ecosystem Functioning Network	MarBEF
Environmental specimens	International Environmental Specimen bank Group	
aDNA	SedaDNA Scientific Society	

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ANNEX II

Further reading and online resources

Biobank Management

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Online resources

Table A10. Sampling protocols.

Marine sampling protocols	https://search.oceanbestpractices.org/
Ocean Genome Legacy Centre protocols	https://ogl.northeastern.edu/wp-content/uploads/2021/09/All-OGL-Sampling-Protocols_2021-9-3.pdf
EAZA Biobank Aquatic Animal Sampling Protocol	https://www.eaza.net/assets/Uploads/Biobank/Biobank-docs/2021/EAZA-Biobank-aquatic-sampling-protocol-final.pdf
Protocols and data for environmental samples	https://www.umweltbundesamt.de/en/topics/chemicals/iesb-specimen-banks/protocols-data
Guidelines for the Handling of Cutting Material, Live Plants and Seeds	https://www.anbg.gov.au/cpbr/herbarium/collecting/live-material.html
Collecting Environmental DNA Samples	https://www.fisheries.noaa.gov/science-blog/collecting-environmental-dna-samples-2022-spring-ecosystem-monitoring-survey
Collecting and preserving insects and mites: techniques and tools	https://www.ars.usda.gov/ARSUserFiles/80420580/CollectingandPreservingInsectsandMites/collpres.pdf
JEMU (Joint Experimental Molecular Unit) protocols	http://jemu.myspecies.info/node/4601

Table A11. SOPs.

National Repository of fish cell line	https://mail.nbfgr.res.in/nrfc/sop.php

Table A12. Genetic resources.

GENRES – System Information for Genetic Resources	www.genres.de/en/
Genesys – Plant Genetic Resources for Food and Agriculture	https://www.genesys-pgr.org/

Table A13. Ethics.

International Treaty on Plant Genetic Resources for Food and Agriculture	https://www.fao.org/plant-treaty/en/
3Rs principles in Wildlife research	https://3rswildlife.info/
Human Tissue Act	www.hta.gov.uk/guidance-professionals

Table A14. Plant regulations and protection.

USDA-APHIS	www.aphis.usda.gov/
European Plant Protection Organization	www.eppo.org
Biosecurity Australia	www.daff.gov.au
Australia Quarantine and Inspection Service	www.aqis.gov.au
Biosecurity New Zealand	www.biosecurity.govt.nz
MAF Quarantine Service	www.maf.govt.nz/mafnet
Plant Health Division of the Canadian Food Inspection Agency	www.inspection.gc.ca
The Plant Health Guide for Importers	www.defra.gov.uk
National, international, and regional plant protection organizations	www.ippc.int/en/

Table A15. Organism´s nomenclature.

Consortium of Pacific Northwest Herbaria	https://www.pacificherbaria.org/
Global Plants Initiative	https://plants.jstor.org
Index Fungorum	https://www.indexfungorum.org/names/names.asp
MycoBank	https://www.mycobank.org/
Catalogue of Life	https://www.catalogueoflife.org/

Table A16. Biodiversity data online.

VerNet	http://vertnet.org/
GBIF – Global Biodiversity Information Facility	https://www.gbif.org/
NBN Atlas	https://nbnatlas.org/
Freshwater Biodiversity Data Portal	https://data.freshwaterbiodiversity.eu/
Global Observation and Biodiversity Information Portal	https://globil.panda.org/
World Flora Online (WFO)	http://www.worldfloraonline.org/
International Plant Names Index (IPNI)	https://www.ipni.org/
World Register of Marine Species (WoRMS)	https://www.marinespecies.org/
AlgaeBase	https://www.algaebase.org/


SYNTHESYS+
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a DiSSCo project