Cryopreservation

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2023

Corrales, C, Leliaert, F, Forrest, L, Martín, M P, Vandelook, F, Thines, M, Poczai, P, Kahila, G, Mulcahy, D, Haring, E, Krukenhauser, L, Mackenzie-Dodds, J, Nagel, M, Ballesteros, D & Astrin, J J 2023, Cryopreservation. in C Corrales & J J Astrin (eds), Biodiversity Biobanking – a Handbook on Protocols and Practices. Pensoft Publishers, pp. 97–113. https://doi.org/10.3897/ab.e101876

http://hdl.handle.net/10138/357562
10.3897/ab.e101876

CHAPTER 5
Cryopreservation

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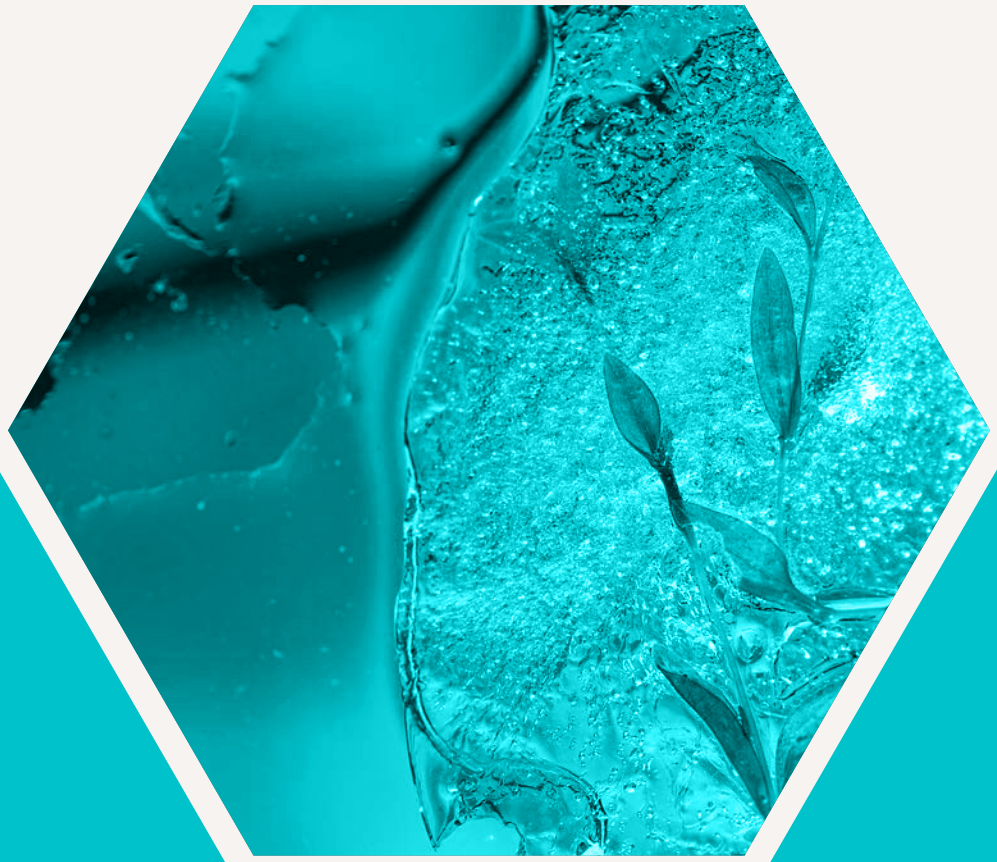

Introduction

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.