Chapter 32

Cryopreservation of Algae

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Abstract

Cryopreservation has been successfully used in the banking and maintenance of cultures of microorganisms, from bacteria to yeasts, since the onset of cryobiology. Biobanking of marine biological resources is crucial for development of scientific knowledge as researchers rely on guaranteed access to reliable, stable resources. Culture collections play a key role in the provision of marine biological resources as they ensure long-term ex situ storage of biological resources that are made available for public and private sector research and education. In this chapter, we provide protocols for cryopreservation of different types of algae cultures.

Key words Microalgae, Macroalgae, Cryopreservation

1 Introduction

Cryopreservation has been successfully used in the banking and maintenance of cultures of microorganisms, from bacteria to yeasts, since the onset of cryobiology. For microalgae, cryopreservation has been increasingly widely applied in recent decades, providing an alternative to the continuous culture of strains [1, 2], saving time and space, and increasing the capacity of collections while avoiding the morphological and physiological modifications that can occur during continuous active culture. While there is no such a thing as a general cryopreservation protocol that can be successfully applied to all microalgal types with guaranteed survival [2–6], there is a more or less standardized method that is routinely applied (notably by culture collections) across large sections of microalgal diversity. For microalgae, cryopreservation success is influenced by many parameters, such as type of strain [7], cell size and cell form [8, 9], culture age [10], and presence of gas vacuoles [11–13]. Cryopreservation protocols can be specifically optimized for each species or strain, but many microalgae have still never been successfully cryopreserved (cryo-recalcitrant species), including, for

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example, most dinoflagellates, some cyanobacteria, and most cryptophytes, with no clear patterns on how to cryopreserve specific groups having been reported. While cryopreservation has been used for freshwater and marine microalgae for decades, macroalgae have only more recently been added to culture collections and therefore represent a new challenge in the field, with several reports of successful cryopreservation in recent years.

Biobanking of marine biological resources is crucial for development of basic scientific knowledge but also for research and development, for example, to discover new biomolecules or processes. Most of these applications rely on guaranteed access to reliable, stable resources. Culture collections ensure long-term ex situ storage of biological resources that are made available for public and private sector research and education. Culture collections play a key role in the provision of marine biological resources, notably by providing information on taxonomic identity, provenance, culture conditions, and sometimes also biological characteristics. In Europe, there are several large algal culture collections, for both marine and freshwater algae, including the Culture Collection of Algae and Protozoa (CCAP) (UK), Sammlung von Algenkulturen Göttingen (SAG) (Germany), the Roscoff Culture Collection (RCC) (France), and the Banco Español de Algas (BEA) (Spain). Outside Europe, the main collections include the National Center for Marine Algae and Microbiota (NCMA) (USA), the Culture Collection of Algae at the University of Texas at Austin (UTEX) (USA), the NIES Microbial Culture Collection (Japan), and the Australian National Algae Culture Collection (ANACC) (Australia). The majority of these culture collections now implement efforts to cryopreserve their culture holdings, with examples of the methods that are applied given below.

2 Materials

1. General laboratory equipment: laminar flow cabinet and appropriate facilities for following sterile microbiological practice.
2. Freezers and cooling equipment: either a passive freezing device (e.g., "Mr. Frosty" from Fisher Bioblock that uses isopropyl alcohol and holds 18 tubes or "CoolCell Lx" from BioCision that does not require alcohol) or a controlled rate freezer (suppliers include Planer UK, CryoLogic, AU, GRANT, Fisher Bioblock) (*see* **Note 1**). Large capacity (several hundred liters) liquid nitrogen cryogenic tanks are generally used for storing samples (either in the liquid or in the vapor phase) (*see* **Note 2**) and a small liquid nitrogen Dewar (4–10 L) for transporting/distributing liquid nitrogen during the freezing protocol.

3. Consumables: sterile culture flasks, cryo-tubes or straws, pipettes, boxes, or canisters for storing tubes/straws, respectively.
4. Safety equipment: lab coat, cryo-gloves, protective eyewear, long forceps, and tweezers (*see* **Note 3**).
5. Post-thaw: a water bath (30–35 °C) for thawing; either an inverted microscope for verifying cell viability or a fluorescent microscope if using a vital stain.
6. Appropriate sterile culture media for incubation before and after cryopreservation and to prepare cryoprotective solutions (e.g., dimethyl sulfoxide, glycerol, sorbitol, polyvinylpyrrolidone (alone and in mixtures)).

3 Methods

3.1 Standard Method for Cryopreserving Microalgae (Used at the RCC and CCAP)

Cultures should be in late exponential or early stationary phase (i.e., dense but healthy) when harvested for cryopreservation. All culture handling should be conducted in a laminar flow cabinet, using sterile consumables and sterile culture medium (the same medium used for routine maintenance of the strain). It is very important to record detailed and accurate information throughout the cryopreservation process, and the use of cold-resistant printed labels on all cryo-tubes is recommended.

1. Concentrate the algae by decantation or by gentle centrifugation to obtain the highest possible density.
2. Cryopreserve using 10% (v/v) dimethyl sulfoxide (DMSO): add 1 mL of concentrated DMSO to 9 mL of culture in a sterile Falcon tube (maintain at the same temperature as the original culture and at low light intensity). Alternatively prepare a 20% (v/v) DMSO solution in sterile culture medium and dilute 1:1 with the culture in a sterile Falcon tube.
3. Allow the mixture to equilibrate for 5 min, and then aseptically put 1 mL into each cryo-tube (total equilibration time 10–15 min).
4. Load cryo-tubes into the controlled rate freezer (*see* **Note 4**) and initiate the following program: starting temperature equivalent to temperature at which original culture is maintained, cooling at 1 °C/min down to −40 °C, hold at −40 °C for 5 min.
5. Transfer cryo-tubes directly into a recipient containing liquid nitrogen taking appropriate safety precautions (*see* **Note 5**).
6. Transfer tubes into plastic boxes for storage in liquid nitrogen and/or −150 °C freezer.
7. For thawing, transfer the cryo-tubes from the liquid nitrogen or −150 °C freezer to a warm water bath (30 °C) and leave

them until all ice melts, avoiding swirling the sample too much to prevent mechanical damage. Wipe each tube with 70% (v/v) ethanol prior to further manipulation. Aseptically empty the contents of each tube into a sterile culture flask and dilute by adding 20 mL of fresh culture media (equivalent to the medium used for the original culture).

8. Incubate at the same temperature as the initial culture. For the first 24 h, it is recommended to incubate in the dark (by covering flasks with aluminum foil) to allow for a gradual reactivation of photosynthetic mechanisms. **Step 7** is also advised to be performed under low light.
9. Viability assays: reanimation success can be assessed simply by visually checking the color of the culture and periodically examining cells under the inverted microscope over time (typically several weeks). Cryopreserved cultures typically exhibit a relatively long lag phase before reaching exponential growth and may therefore take several weeks to recover normal growth.

In order to illustrate the efficiency of this method, statistics for employment of the method in recent years by the RCC are presented below. Since 2011, in total this method has been applied to 1765 strains (almost exclusively marine) from the RCC, with 1551 (88%) having been successfully reanimated. This high success rate in part reflects the fact that in most cases, cryopreservation has been applied to species/strains that are expected to be resistant. Of successfully cryopreserved strains, the majority are green algae (Chlorobionta) (399), cyanobacteria (332), alveolate parasites (188), diatoms (184), haptophytes (170), and bacteria (143). Ten dinoflagellate strains have been successfully cryopreserved. Of the strains for which cryopreservation was not successful, the majority are diatoms (94), green algae (Chlorobionta) (57), and dinoflagellates (13). In terms of percentage of total strains of different groups held in the collection that have been cryopreserved, cyanobacteria, green algae, bacteria, and alveolate parasites are all above approximately 40% (*see* Fig. 1). Note that in many cases, the percentage is low because cryopreservation has not yet been attempted for many strains in the group.

A full list of species that have successfully been cryopreserved using the above method is given in Table 1 (note that in many cases, there are species for which cryopreservation was successful for some strains but not others). Diatoms can be used as a test case to investigate the factors that may influence cryopreservation success. In total, 278 diatom strains have been tested at the RCC, 184 (66%) of which have been successfully reanimated after cryopreservation. There is considerable variability between taxonomic groups (genera or classes) within the diatoms in cryopreservation

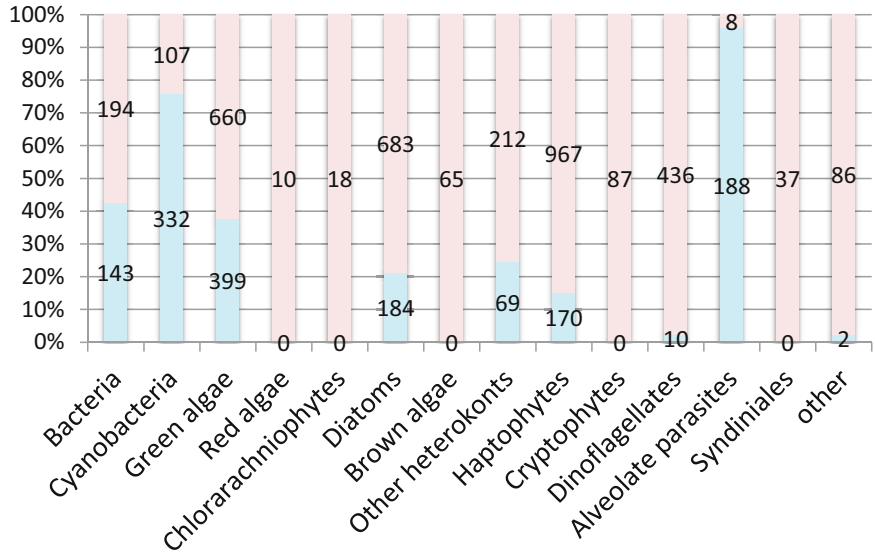

Fig. 1 Quantity and proportion of strains of different prokaryotic/eukaryotic groups cryopreserved at the RCC (blue, successfully cryopreserved; pink, not cryopreserved)

success (*see* Table 2a, b). In terms of geographic origin, approximately two thirds of temperate and polar strains cryopreserved successfully, whereas almost all tropical strains (albeit from a lower total number) grew after thawing (*see* Table 2c). In terms of estimated cell size, approximately two thirds of the small (<1000 μm^3) and intermediate (1000–2500 μm^3) categories cryopreserved successfully, whereas few of the larger (>2500 μm^3) category survived cryopreservation (*see* Table 2d). Cell size therefore appears to be a key factor in cryopreservation success, with cold-adapted (polar) strains apparently not showing an increased resistance to cryopreservation.

3.2 Cryopreservation of Microalgae of Interest in Aquaculture: Chaetoceros neogracilis *(RCC2278),* Nannochloropsis gaditana *(CSIC-ICMAN Owned), and* Rhodomonas lens *(CCMP 739)*

1. The culture should be either in exponential growth or in early stationary phase when harvesting for cryopreservation. Concentrate the algae by decantation when possible or by gentle centrifugation to obtain the highest possible density. All handling should be conducted in a laminar flow cabinet, using sterile materials and media.
2. All three species can be cryopreserved using a 15% (v/v) DMSO solution prepared in sterile culture medium. This solution should be prepared at twice the desired final concentration to allow for a 1:1 dilution factor in **step 3**.
3. Using 2 mL cryo-tubes, add 1 mL of culture and stepwise add 1 mL of cryoprotectant solution in ten steps of 100 μL (1 min apart) to allow for gentle cellular equilibration at room temperature (18–20 °C). This step is preferably conducted under low light. Allow the cells to equilibrate for 5 more minutes while you close the cryo-tubes. Total equilibration time 15 min.

Table 1
List of species successfully cryopreserved by the RCC using the standard protocol

Group	Class	Species	Group	Class	Species
Cyanobacteria	*Cyanophyceae*	*Synechococcus* sp.	Green algae	Chlorophyceae	*Chlamydomonas* sp.
Diatoms	Bacillariophyceae	*Amphora* sp.			*Chlamydomonas concordia*
		Biremis lucens			*Microglena reginae*
		Cylindrotheca sp.		Chloropicophyceae	*Chloropicon* sp.
		Entomoneis sp.			*Chloropicon laureae*
		Nitzschia sp.			*Chloropicon mariensis*
		Nitzschia closterium			*Chloropicon maureeniae*
		Opephora sp.			*Chloropicon primus*
		Pseudo-nitzschia sp.			*Chloropicon roscoffensis*
		Pseudo-nitzschia subcurvata			*Chloropicon sieburthii*
		Pseudo-nitzschia seriata			*Chloroparvula* sp.
		Staurosira sp.			*Chloroparvula japonica*
	Mediophyceae	*Chaetoceros* sp.		Mamiellophyceae	*Ostreococcus mediterraneus*
		Chaetoceros calcitrans			*Bathycoccus oceanicus*
		Chaetoceros gelidus			*Bathycoccus prasinos*
		Chaetoceros neogracilis			*Ostreococcus* sp.
		Minidiscus sp.			*Ostreococcus lucimarinus*
		Minidiscus comicus			*Ostreococcus tauri*
		Minidiscus spinulatus			*Micromonas* sp.
		Minidiscus variabilis			*Micromonas bravo*
		Minutocellulus sp.			*Micromonas commoda*
		Minutocellulus polymorphus			*Micromonas pusilla*
		Skeletonema cf. *costatum*		Palmophyllophyceae	*Prasinococcus* sp.
		Skeletonema sp.			*Prasinococcus* cf. *capsulatus*
		Skeletonema japonicum			*Prasinoderma* sp.
		Thalassiosira sp.			*Prasinoderma coloniale*
		Thalassiosira minima		Picocystophyceae	*Picocystis salinarum*
		Thalassiosira pacifica		Prasinophyceae	*Pycnococcus* sp.
		Thalassiosira profunda			*Pycnococcus provasolii*
		Thalassiosira pseudonana		Trebouxiophyceae	*Picochlorum* sp.
Other heterokonts	Pelagophyceae	*Pelagococcus* sp.			*Picochlorum atomus*
		Pelagomonas calceolata			*Picochlorum costavermella*
	Eustigmatophyceae	*Microchloropsis gaditana*	Alveolates	Dinophyceae	*Symbiodinium* sp.
		Nannochloropsis granulata			*Symbiodinium kawagutii*
Haptophytes	Prymnesiophyceae	*Dicrateria* sp.			*Symbiodinium microadriaticum*
		Dicrateria rotunda			*Symbiodinium psygmophilum*
		Emiliania huxleyi			*Symbiodinium voratum*
				Perkinsea	*Parvilucifera* sp.
					Parvilucifera infectans
			Viruses	Phycodnaviridae	*Prasinovirus* sp.

Table 2
(a) Cryopreservation of diatom genera at the RCC using the standard protocol detailed in Note 1. (b) Cryopreservation of diatom classes/orders at the RCC using the standard protocol detailed in Note 1. (c) Success rate of diatom cryopreservation at the RCC according to geographic origin. (d) Success rate of diatom cryopreservation at the RCC according to cell volume

(a)

Genus	***Successful***	***Not successful***
Thalassiosira	36	25
Minidiscus	32	28
Nitzschia	31	0
Skeletonema	22	3
Chaetoceros	16	8
Minutocellus	7	0
Cylindrotheca	5	0
Guinardia	0	6
Other/unknown	35	24
	184	*94*

(b)

Class	***Order***	***Successful***	***Not successful***
Mediophyceae	Thalassiosirales	89	56
	Chaetocerotales	17	8
	Cymatosirales	19	6
Bacillariophyceae	Bacillariales	38	0
Coscinodiscophyceae	Rhizosoleniales	0	6

(c)

Origin	***Successful***	***Not successful***	***% Successful***
Temperate	137	77	64%
Polar	30	16	65%
Tropical	17	1	94%

(d)

Category	***Cell volume***	***Successful***	***Not successful***	***% Successful***
Small	$<1000\ \mu m^3$	115	51	69%
Medium	$1000–2500\ \mu m^3$	67	31	66%
Large	$>2500\ \mu m^3$	2	12	14%

4. *Chaetoceros neogracilis* can be successfully cryopreserved using a passive freezer (*see* **Note 6**) with the tubes placed in the rack that fits in the bath of isopropyl alcohol. The passive freezer is placed in the −80 °C freezer for 100 min, and the isopropyl

alcohol (and therefore the samples) will cool down at an approximate rate of 1 °C/min. After 100 min, the samples are at −80 °C and can be directly transferred to liquid nitrogen for storage.

5. *Nannochloropsis gaditana* and *Rhodomonas lens* can also be cryopreserved using a passive freezer, but in our experience, survival and fitness of these species are improved when cryopreserved using a controlled rate freezer (*see* **Note 4**) with the following cooling ramp: 1 °C/min down to −10 °C, hold at −10 °C for 1 min and cool down at 3 °C/min to −40 °C, holding this temperature for a couple of minutes before transferring the straws to liquid nitrogen for storage.
6. Storage in liquid nitrogen requires handling of samples while immersed in liquid nitrogen, and there are therefore several security precautions that should be applied (*see* **Note 5**). Cryotubes (and/or straws) can then safely be transferred using the large forceps or tweezers into the storage containers. Storage containers can be cardboard or plastic boxes or canisters (depending on whether cryo-tubes or straws are used).
7. For thawing, transfer the cryo-tubes/straws from the liquid nitrogen to a warm water bath (35 °C) and leave them until all ice melts, avoiding swirling the sample too much to prevent mechanical damage. This step can either be conducted with sterile water inside the flow cabinet or outside, in which case each tube/straw should be wiped with 70% ethanol prior to further manipulation. Empty one tube (or four to eight straws) into sterile 10 mL test tubes and dilute the cryoprotective agent by adding fresh culture media in ten steps of 200 μL, one min apart. After this 1:1 stepwise dilution, top the vial up to 5 mL for incubation.
8. For the first 24 h, it is recommended to incubate under low light intensity to allow for a gradual cell reactivation of photosynthetic mechanisms. **Step 7** is also recommended to be performed under low light.
9. Viability assays: success can be assessed simply by examining cells under the inverted microscope, by measuring cell density (manual cell counting, flow cytometry quantification, measurement of in vivo fluorescence) over time (typically a few weeks), or by using vital stain. Cryopreserved cultures typically exhibit a quite long lag phase before reaching exponential growth and providing an active culture than can be scaled up to larger volumes.

3.3 Cryopreservation of Hypersaline Chlorophyte Strains of Interest in Biotechnology: Dunaliella salina, D. minuta, D. tertiolecta, D. velox, D. viridis, and D. bioculata

1. The strains cryopreserved are listed in Table 3. Cultures should be healthy and free from contamination and in the late exponential growth phase (determined by daily cell counts in the period preceding cryopreservation).
2. Strains should be cryopreserved in the same media in which they are grown. For these strains, this routinely has 50 g/L of commercially available sea salts added. All media used should be sterilized by autoclave or filter sterilization before use. All work should be conducted in a laminar flow clean cabinet and all equipment cleaned with 70% ethanol before placing into the laminar flow cabinet. All plastics used are commercially sterilized, and pipette tips have filters to avoid any contamination from the pipette.
3. All strains outlined above can be cryopreserved in 10% (v/v) DMSO as a cryoprotectant. This final concentration is obtained by making an initial solution of 20% (v/v) DMSO in growth media. This is then filter sterilized using a 0.22 μm syringe filter and added to an equal volume of the microalgae culture. It is important to note that the initial addition of DMSO to media heats the solution; therefore, adequate time

Table 3
Hypersaline chlorophyte strains of interest in biotechnology: *Dunaliella salina*, *D. minuta*, *D. tertiolecta*, *D. velox*, *D. viridis*, and *D. bioculata*

Strain number	*Dunaliella* culture	Strain number	*Dunaliella* culture
PLY SA-1	*D. viridis*	PLY DF-8	*D. salina*
PLY SA-2	*D. viridis*	PLY DF-11	*D. salina*
PLY SA-3	*D. salina aureus*	PLY DF-12	*D. salina*
PLY SA-4	*D. salina aureus*	PLY T-32	*D. bioculata*
PLY SA-5	*D. velox*	PLY T-34	*D. minuta*
PLY SA-6	*D. velox*	PLY T-36	*D. salina aureus*
PLY DF-15	*D. salina rubeus*	PLY T-37	*D. salina aureus*
PLY DF-17	*D. salina*	PLY T-41	*D. salina aureus*
PLY SA-7	*D. bioculata*	PLY T-68	*D. salina aureus*
PLY DF-1	*D. salina*	PLY T-74	*D. salina*
PLY DF-3	*D. salina*	PLY T-75	*D. minuta*
PLY DF-4	*D. salina*	PLY T-76	*D. minuta*
PLY DF-7	*D. salina*	PLY T-77	*D. minuta*
		UTEX 999	*D. tertiolecta*

should be allowed for the solution to return to room temperature before adding to the cell culture.

4. One milliliter of this culture/cryoprotectant mixture is then aliquoted into 1.8 mL cryogenic vials and placed into a passive freezing container (*see* **Note 6**) to freeze at a rate of 1 °C/min (we would recommend freezing several vials so that viability can be tested by thawing while some samples still remain frozen for long-term storage). This should then remain at room temperature for 10 min in order for the cryoprotectant to penetrate the cells. The passive freezing container is then placed into a −80 °C freezer for 100 min.
5. After this time, the container is removed, and the cryovials plunged individually into liquid nitrogen (*see* **Note 5**) using large forceps and personal protective equipment (PPE). The vials are then transferred either into a −150 °C ultra-freezer or into liquid nitrogen storage.
6. When thawing the algae, it is important to expose the samples to as little light as possible. To thaw, the vials are removed from the freezer/liquid nitrogen and agitated gently, one at a time, in a 40 °C water bath until all ice crystals are gone (no longer than 2 min) (*see* **Note 7**). The thawed vial is then quickly cleaned with 70% ethanol and transferred to the laminar flow cabinet. The contents are then transferred, by pipetting, into 20 mL of growth media in a sterile growth flask with a vented lid.
7. This is then placed into a dark box and placed at growth temperature. The cultures are left in the dark for 24 h. After this time, the lid is gradually opened to allow some light in. After 48 h of thawing, the cultures can be removed and placed at the light intensity they are normally grown at.
8. Viability should be assessed microscopically although it is worth noting that it can take up to 6 weeks for a strong culture to establish.

3.4 Cryopreservation of Macroalgae

In the Culture Collection of Algae and Protozoa (CCAP) laboratory in recent years, a range of macroalgal species have successfully cryopreserved using slight modifications of the standard two-step method used for microalgae (*see* Table 4 and Fig. 2). To successfully cryopreserve macroalgae, the starting material must be relatively dense, healthy, and growing well with minimal bacterial contamination. Large thalli and clumps of filamentous species should be carefully dissected into small pieces, taking care not to introduce contamination [14–16]. In some cases, the addition of an extracellular membrane impermeable cryoprotectant is required such as sorbitol or polyvinylpyrrolidone (PVP).

Table 4
Summary of species and conditions for the cryopreservation of macroalgae

Species	Cryoprotectant	Cooling rate	Cooling method	References
Porphyra umbilicalis	5% DMSO/5% PVP	1 °C/min	Controlled rate freezer	Kuwano et al. [14]
Ectocarpus sp.	10% DMSO/9% sorbitol	1 °C/min	Controlled rate freezer	Heesch et al. [15]
Ulva lactuca	20% Glycerol	1 °C/min	Mr. Frosty	Pers. comm. C.N. Campbell
Saccharina latissima	10% DMSO	1 °C/min	Controlled rate freezer	Visch et al. [16]
Arctic brown seaweeds[a]	5% DMSO	1 °C/min	Controlled rate freezer	Pers. comm. C.N. Campbell

[a]Polar strains of five brown seaweeds were successfully cryopreserved (personal communication CCAP) maintaining all media and materials at the normal growth temperature of 2 °C in the initial stage and post-thaw. The frozen strains included CCAP 1300/1 *Agarum clathrum*, CCAP 1306/45 *Desmarestia aculeata*, CCAP 1306/46 *Desmarestia* sp., CCAP 1334/1 *Stictyosiphon tortilis*, and CCAP 1333/1 *Petalonia* sp.

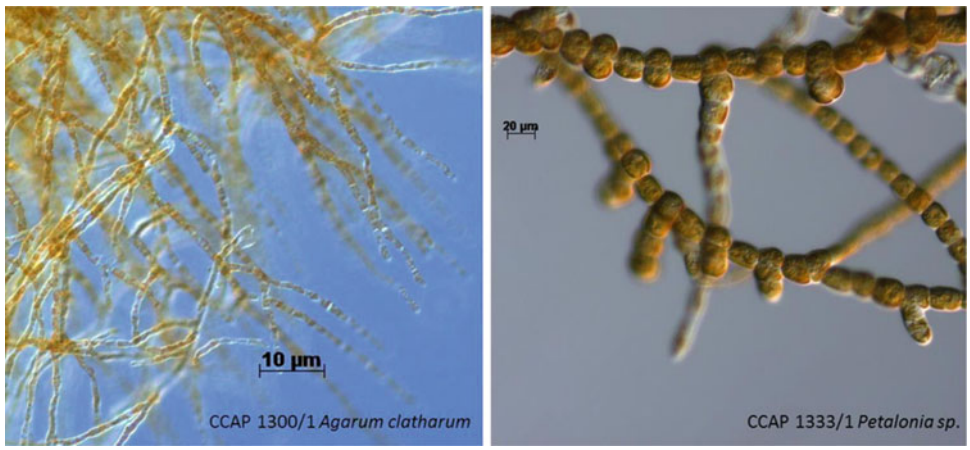

Fig. 2 Examples of macroalgae successfully cryopreserved at the CCAP

3.5 Recalcitrant Species

There are many types of algae that do not survive cryopreservation, termed "cryo-recalcitrant" strains. As experiments to attempt to freeze them fail to provide positive results, these tend not to be reported in the literature. In the CCAP, experiments were carried out to attempt to cryopreserve three examples of typically recalcitrant taxa and to observe the effect of applying cryoprotectants. We chose two cryptophyte strains, the freshwater *Cryptomonas* CCAP 979/44 and the marine *Rhodomonas* CCAP 995/2, as well as the marine dinoflagellate *Lingulodinium polyedra* CCAP 1121/5.

Table 5
Toxicity assays of different cryoprotective agents examined for three recalcitrant species prior to cryopreservation

	Exposure time		Control	MeOH 5%	MeOH 10%	MeOH 15%	DMSO 5%	DMSO 10%	DMSO 15%	DMSO 20%	Glycerol 5%	Glycerol 10%	Glycerol 15%	Glycerol 20%	Glycerol 15% DMSO 5%	Glycerol 20% DMSO 5%	Glycerol 15% DMSO 10%	Glycerol 20% DMSO 10%	Glycerol 15% MeOH 5%	Glycerol 20% MeOH 5%	Glycerol 15% MeOH 10%	Glycerol 20% MeOH 10%	DMSO 5% MeOH 5%	DMSO 10% MeOH 5%	DMSO 5% MeOH 10%	DMSO 10% MeOH 10%
Cryptomonas	10 min	Motile	√	√	√	√	-	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	-	-	x
		Content	√	√	√	√	√	√	√	√	x	x	x	x	x	x	x	x	x	x	x	x	√	√	√	√
	30min	Motile	√	√	√	√	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	-	-	x
		Content	√	√	√	√	√	√	-	x	x	x	x	x	x	x	x	x	x	x	x	x	√	√	√	√
	1h	Motile	√	√	√	√	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	x	-	x
		Content	√	√	√	√	√	√	-	x	x	x	x	x	x	x	x	x	x	x	x	x	√	√	√	√
	24h	Motile	√	√	√	√	√	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	x	√	x
		Content	√	√	√	√	√	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	x	√	x
	96h	Motile	√	√	√	√	√	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	x	x	x
		Content	√	√	√	√	√	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	√	x	x	x
Rhodomonas reticulata	10 min	Motile	√	√	√	√	-	-	-	x	√	x	x	x	x	x	x	x	-	x	x	x	-	x	x	x
		Content	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	x	√	√	√	x	√	√	√	√
	30min	Motile	√	√	√	√	√	-	x	√	x	x	x	x	x	x	x	x	x	x	x	x	-	-	√	x
		Content	√	√	√	√	√	√	√	√	√	√	-	√	√	√	x	-	√	√	x	√	√	√	√
	1h	Motile	√	√	√	√	-	-	-	x	-	x	x	x	x	x	x	x	x	x	x	-	-	√	x
		Content	√	√	√	√	√	√	√	√	√	√	√	√	√	√	-	x	√	√	√	x	√	√	√	√
	24h	Motile	√	√	x	x	√	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	√	√	√	x
		Content	√	√	√	√	√	√	√	x	√	x	x	x	√	√	√	x	√	x	√	x	√	√	√	√
	96h	Motile	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Content	√	√	√	√	√	x	x	x	x	√	x	x	x	x	x	x	x	x	x	x	x	√	√	√
Lingulodinium polyedra	10 min	Motile	√	√	√	√	√	-	x	x	√	x	x	x	x	x	x	x	x	x	x	x	√	x	√	x
		Content	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	30min	Motile	√	√	√	√	√	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	x	-	x
		Content	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	1h	Motile	√	√	√	√	√	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	-	x	-	x
		Content	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	24h	Motile	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Content	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	96h	Motile	cyst	x	x	x	cyst	cyst	x	x	cyst	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Content	√	x	x	x	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√

√ positive result
x negative result
- Not done

Different concentrations of methanol, DMSO, and glycerol and combinations of these were added to cultures of the three strains, and they were observed for motility and cell integrity at periods up to 96 h. All strains remained motile and intact after 2 h in all concentrations of methanol. After 24 h, all *Lingulodinium* cells lost motility in every cryoprotectant; however, the cells appeared to be intact and pigmented in all treatments up to 96 h. The results are presented in Table 5.

Choosing what appeared to be the optimal cryoprotectant concentrations, we then cryopreserved all our strains using the two-step protocol freezing at 1 °C/min. On post-thaw microscopic examination, all cryptophyte cells had lysed and were nonviable. Many of the dinoflagellate cells appeared to be intact and pigmented but failed to regain motility or to divide.

Another recalcitrant species of interest is the dinoflagellate *Alexandrium minutum*. At the Toralla Marine Science Station (University of Vigo Spain), we have focused time and effort on attempting to develop a cryopreservation protocol for this

dinoflagellate which is commonly related with toxicity in red tides in Galicia and elsewhere. The techniques used were a two-step traditional cryopreservation, slow cryopreservation using passive freezing systems, vitrification, vitrification with ultra-fast laser warming, and encapsulation-vitrification. We tested both DMSO and glycerol at 10% and 15% as cryoprotectants. After cryopreservation, post-thaw studies were made to observe culture viability (recovery and density counts to compare them with controls). *A. minutum* showed extreme sensitivity to cryoprotectant exposure (even stepwise). We observed that dehydration events are lethal to this species and it did not survive any of the cryopreservation treatments. After vitrification with ultra-fast laser warming, we have observed that the cells were intact, the membrane was not detached, and the cell looked morphologically alive. However, cells failed to regain motility or divide. At the Marine Biological Association in Plymouth, UK, the dinoflagellate strains PLY 701 *Prorocentrum triestinum*, PLY 626 *Prorocentrum micans*, and PLY 632 *Scrippsiella trochoidea* failed to cryopreserve successfully using various methods. The benthic dinoflagellate strains, PLY 440 *Amphidinium* sp., PLY 450 *Amphidinium carterae*, and PLY 734 *Amphidinium carterae*, however, were cryopreserved and reanimated successfully. This is supported by similar findings for this genus at the CCAP.

3.6 Cryopreservation, Long-Term Storage, and Viability

Conservation in liquid nitrogen (−196 °C) will ensure the indefinite preservation of cells, as has been reported for many cell types [e.g., 17–19]. Ultralow temperature (−150 °C) freezers can also be used for storing cryopreserved samples, but appropriate backup systems are required in case of power failure. Once cells are stored at these temperatures (and maintained at temperatures below the glass transition temperature), their viability is theoretically only affected by background radiation, which at normal levels will take 2000 years to become a hazard to stored cells [20]. The viability of cryopreserved microalgae stored in conventional −80 °C or −20 °C freezers is suboptimal and limited in time compared to storage in liquid nitrogen [21].

When undertaking routine cryopreservation, culture collections usually qualitatively assess post-thaw viability using a combination of short-term and long-term factors. Immediately post-thaw, cell motility and morphology can easily be assessed under the microscope. Subsequently, culture recovery can be assessed by simple visual assessment of the color of the culture flask. Quantitative methods to assess post-thaw viability include cell counting (manual counting, flow cytometry, in vivo fluorescence) or use of a vital stain such as DAPI.

4 Notes

1. If a passive device is used, a −80 °C freezer is required.
2. A −150 °C freezer can also be used.
3. All manipulations should be carried out in a well-ventilated laboratory with sensors to monitor oxygen levels.
4. Delicate or more complex microalgae have a better fitness post-thaw when cryopreserved using a controlled rate freezer that allows for the application of a custom-made cooling ramp, usually employing faster cooling rates.
5. If you need further information about liquid nitrogen management and safety precautions, how to store your samples safely, or how to track your cryopreserved samples, you should check the ISBER Best Practices: Recommendations for Repositories [22].
6. Passive freezers are containers with a lid that hold a deposit for the cooling solution (pure isopropyl alcohol) and a holder for the vials (1.8 mL cryovials with a silicon ring for safe closing are recommended). There are alternatives in the market made of a thermo-conductive material that does not need alcohol. They are usually quite affordable methods to cryopreserve the most resistant microalgae species when there is a −80 °C freezer available.
7. It is important to note that other vials that are stored alongside the one to be thawed should not be warmed for a prolonged amount of time as even a small increase in temperature can have detrimental effects on the cells. To avoid this, the entire container (e.g., cryobox) can be placed into liquid nitrogen while the vials to be thawed are removed.

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