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GENERAL DATA

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Abstract

The aim of this report if to establish the state of the art of the methodology and accumulated knowledge as a start point for the development of the Work Package coordinated research effort in cryobiology applied to the marine environment, as well as, the establishment of a discussion forum for the collaborating partners.

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1. Introduction

Fundamental to the sustainable exploitation (scientific and biotechnological) of marine resources is our capacity to maintain them *ex situ*. Only cryopreservation, with ultra-low temperature storage provides the technical possibility of long-term conservation of resources as diverse as echinoderm embryos, macro/micro-algae or fish germplasm. Cryobiology research on marine organisms is still in its infancy when compared to terrestrial systems. Moreover, there are many marine organisms for which there is a lack of data regarding cryopreservation. The scarcity of effective cryopreservation methodologies for key cell types such as germ cells and embryos is limiting the development and exploitation of this sector of science, a clear need exists to have reliable methodologies as well as strong research activity in marine cryobiology. The advances made in this JRA will form a major breakthrough that will generate immense benefits not only for research, but also directly on economic activities including: aquaculture, fisheries management, environmental monitoring, biomedicine and sustainable biotechnological exploitation of marine biological resources.

Building on the partner's experience in cryopreservation, culture collections/ Biological Resource Centre management, biobanking of marine organisms, cell and fixed specimen preservation, and through the utilization of the findings of previous EU funded projects we can increase the knowledge in marine cryobiology exponentially and develop cryopreservation protocols for many previously under-exploited cell types. Increasing the critical mass of knowledge in this preservation technology, will result in the increase of fundamental and applied uses. The availability of cryopreserved marine embryos or cells will complement many lines of research already developed or in development within the European marine research community: genetics, reproductive biology, conservation, ecotoxicology, aquaculture, biofuel, green energy, biogeochemistry, marine biotechnology and medical research. Furthermore, the capacity to conserve microbial consortia and mutant libraries will have wide-reaching implication for blue-skies and applied research as well as biotechnology.

The Uniqueness of this service will benefit the EMBRC and the whole marine biological community by providing means and highly qualified staff that will develop cryopreservation protocols for marine organisms and that will offer biobanking services and a biobanked portfolio of species. Furthermore, it will provide a platform for TNA development of methodologies for taxa of specific interest to individual groups/organisations, as was previously trialled by the ASSEMBLE project.

2. Objectives

WP JRA2 Cryopreservation of Marine Organisms objective is to address a constraint in the exploitation of marine genetic and biological resources, namely current paucity of capability to conserve these resources ex situ with guaranteed genetic, phenotypic and functional stability. The JRA will develop robust, reproducible cryopreservation methodologies for various life-stages of a range of marine macro-organisms and currently cryo-recalcitrant microorganisms.

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The results will improve and expand the availability of biological resources for TA at significantly reduced costs.
The aim of this report if to establish the state of the art of the methodology and accumulated knowledge as a start point for the development of the Work Package coordinated research effort, as well as, the establishment of a discussion forum for the collaborating partners.

3. Review of cryobiological methods

3.1 Slow cryopreservation/Traditional Cryopreservation in liquid nitrogen & thawing using controlled-rate freezers

Principles: The freezing point of the external solution will depend on the solution media and the cryoprotectant agents present. Once the cooling starts the external medium will freeze before the cells and water inside will be under its thermodynamic freezing point. Water below its freezing point is defined as supercooled and has higher vapour pressure at a given temperature than the ice outside the cell (Dennison *et al.* 2000, Mazur 2004). As long as this difference in potential remains, water will slowly leave the cell and freeze externally. Concentration of solutes inside the cell will increase and the potential is reduced to reach the thermodynamic equilibrium (the speed of this process depends on the cooling rate and water permeability at a given temperature). Following this process, the cell will slowly dehydrate avoiding the Internal/Intercellular Ice Formation (IIF), which is lethal for the vast majority of biological materials (Mazur 2004). Too slow cooling rates, results in excessive intercellular dehydration and will lead to high concentration of electrolytes that can damage the cells, accompanying a large reduction in cell volume that can produce irreversible membrane deformations. On the other side, too fast cooling rates will not allow cells to dehydrate and intracellular water will become increasingly supercooled. Once reached the intracellular nucleation temperature (the temperature at which ice nucleation of the remaining supercooled intracellular water occurs), IIF will occur (Mazur 1970, Leibo and Mazur 1971, Leibo *et al.* 1974, Mazur 2004, Pegg 2007). Samples are normally stored in liquid nitrogen or liquid nitrogen vapour for conservation, but alternative electrically cooled systems are now employed by many practitioners in the biobanking and assisted reproduction sectors.

Cooling rates: Most commonly used slow cooling/freezing rates vary between 0.5ºC/min to 10ºC/min. In order to follow these cooling rates in a controlled manner this technique uses controlled-rate freezers and the most common volumes of the samples are under 4 ml. In many systems ice nucleation can be induced either mechanically, or by a ”pulse” of rapid cooling, this allows greater control and may reduce the effects of random nucleation in vials at different supercooled temperatures (In theory this may result in differential levels of cryodehydration that could have implications on viability levels)
Thawing: Thawing is usually performed by immersion of samples in a pre-warmed water bath.
Uses: Most used for embryo and larval cryopreservation.

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3.2 Slow cryopreservation using passive freezing systems

Principles: The principles of cryopreservation of slow cryopreservation apply here, in this case the theoretical cooling rate is calculated by the cooling rate of a liquid in which the vials are immersed. Therefore, the cooling rate it is not actively controlled by a device. Instead, the vials with the sample are placed in a container half filled with Isopropyl alcohol and placed in a -80ºC freezer.
The lack of control over the stability of the cooling process as well as the restrained cooling options makes this method suitable only for the most robust of the species and cell types although a cheap alternative (Paredes et al. 2013)
Cooling rates: The isopropyl alcohol cools down at an average 1ºC/min until reaching the freezer temperature. Then the samples could be transferred to liquid nitrogen for storage. **Thawing:** Thawing is usually performed by immersion on a water bath. **Uses:** Common use for non-delicate microalgae cryopreservation

3.3 Cryopreservation in liquid nitrogen vapour and thawing

Principles: This is another method of slow cooling by setting the samples over a floating device over liquid nitrogen surface. The samples will cool down by exposure to the liquid nitrogen vapour for a determined period of time before being plunged into liquid nitrogen for conservation. In order to control the parameter of the cooling rate factors such as: the exposure time, volume of the sample, cm floating above the nitrogen level should be controlled and clearly stated when publishing the results.
Cooling rates: In this case the cooling rate is dependent of the height above the nitrogen, exposure time and volume of the samples. Cooling rates of 30-100ºC/minutes have been reported (Adams et al. 2004). **Thawing:** Thawing is usually performed by immersion on a water bath. **Uses:** Common for sperm cryopreservation for marine invertebrates and fish.

3.4 Vitrification and ultra-fast warming

Principles: In the past decade and a half, there has been considerable clinical interest in the cryopreservation of human oocytes (Fuller *et al.* 2004), which have proved more difficult to preserve by slow freezing methods; thus, interest has shifted somewhat to cryopreserving them by vitrification (Kuwayama *et al.* 2005; Gardner *et al.* 2007). One way to avoid lethal IIF is to cool cells slowly enough so that the freezable water in the cell flows out of the cell and freezes externally (Slow freezing methods), but an alternative option is to avoid intracellular ice formation by vitrifying cellular water and this approach has increasingly become the method of choice. Vitrification involves converting water into a non-crystalline or amorphous glass. To achieve this conversion, the initial view has been that cells have to be immersed in solutions containing a very high concentration of glass-inducing solutes (typically 6 molal, which is a highly toxic concentration) and that they have to be cooled very rapidly (typically at rates >50 000ºC/min). The belief that rapid cooling was essential has been challenged in the last decade (Seki and Mazur 2009, Seki and Mazur 2012, Jin and Mazur 2015). The especially high sensitivity to warming rate strongly suggests that the lethality of slow warming is a consequence of either the crystallisation of intracellular glassy water during warming or the recrystallization during slow warming of small intracellular crystals that had formed during

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cooling (Mazur and Seki 2011). Vitrification and ultra-fast warming by laser (Seki et al. 2014, Jin and Mazur 2015, Mazur and Paredes 2016) or rapid warming by nanoparticles (Khosla et al. 2017) have been producing high survivals and important advances in cell types and species who had been challenging to cryopreserved by the slow cooling methods.

Cooling rates: Over 50,000ºC/minute. Although high survivals can be achieved with slower rates when the warming rate is high enough. **Thawing rates:** Mazur´s ultra-rapid laser system published warming rates for mice embryos of 10^7 ºC/min **Uses:** This method have been used with: mice oocytes and embryos, yeast producing high survivals and zebrafish embryos with a quite hopeful results. This method have never been applied to the cryopreservation of cells from marine origins.

3.5 Encapsulation/vitrification

Principles: In an encapsulation-vitrification protocol, the cells are encapsulated in alginate beads, loaded and dehydrated with a vitrification solution, and or by placing them in a "stream" of sterile air, before rapid immersion in liquid nitrogen.
Cooling rates: Around 200ºC/min. The prior dehydration before the beds are plunged into liquid nitrogen allow the sample to get vitrified at this low cooling rates. **Thawing:** By immersion in culture media (around 250ºC/min)**. Uses:** Mostly it has been used for cryopreservation of plants and freshwater microalgae. There is not a protocol described for the application for this methodology to the marine microalgae, not least because the encapsulating alginate matrix depolymerises due to the high sodium levels in seawater/ marine media.

3.6 Storage

As discussed above, where conditions are maintained at ultra-low temperatures (< -130ºC), prolonged storage at least for decades should have no significant effect on viability of algae (Day et al. 1997). However, temperature fluctuations during storage have the potential to induce freeze-fracture and de-vitrification events in the stored sample. These, in turn, could influence viability levels. Although cryobiological theory would clearly indicate that storage at temperatures where ice crystal growth occurs should result in reduced/ no post-thaw viability (Grout, 1995), there are few studies that substantiate this statement.

It is particularly important that high standards are maintained in facilities handling genetically manipulated organisms and patent depositories where the preserved cells must remain viable for at least 20 years (Budapest Treaty, 1977). Security and stability of stored material is assured through adoption of appropriate management systems to restrict access to authorized personnel, appropriate alarms for nitrogen storage vessels and documented procedures for filling and maintenance of nitrogen storage. Monitoring using temperature alarm systems and auditing to ensure correct maintenance and documentation are also important activities for BRC operation. Accurate records are vital to enable retrieval of the stored ampoules. Proprietary database systems have been specially designed for this purpose, but it is important to select a system which is flexible to the full range of user requirements. It is also sensible to have an up to date hard-copy version, or back-up electronic copies and if possible ensure that amendments to storage records can be made at the storage site to avoid transcriptional errors.

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3.7 Freeze-dry

Principles: Freeze-drying or lyophilisation describe precisely the same process. Operationally freeze-drying may be defined as a controllable method of dehydrating labile products by vacuum desiccation (Adams 2008).

Uses: It has been used successfully to preserve yeasts, fungi and bacteria, including cyanobacteria, but, with the exception of marine bacteria, has not been successfully applied to marine organisms.

4. State of the art

4.1 Marine Invertebrate cryopreservation

The cryopreservation of marine invertebrate organisms has been reduced to a limited number of species and cell types, in contrast to the cryopreservation research on mammals, fish or plants (Paniagua-Chavez *et al.* 2001). Oysters and sea urchins were the initial targets of the first studies (Lanan 1971 on *C. gigas* sperm and Asahina and Takagashi 1977 on *H. pulcherrimus* embryos) but now the number of different species studied exceeds fifty. Despite this, there is still limited work on marine invertebrate cryopreservation. Most publications deal with mollusks, and among them, oysters are the best studied due to their global economic importance. *Crassostrea gigas* represents >60% of the total work published on oysters, followed by *C. virginica*. Together molluscs and echinoids account for >70% of the research effort.
As far as cell types, initially oocytes and sperm seem to be the natural targets for cryopreservation. In fact, successful sperm cryopreservation has been reported for many marine invertebrate species and represents ±50% of the published work. Oocytes had been more challenging, and limited success has been achieved (e.g.Tsai and Chao 1994, Tervit et al. 2005, Salinas-Flores et al. 2008a, Adams et al. 2011a, Yang et al. 2013) to the point that oocyte studies only represent 10% of the published work in the field. Cryopreservation of embryos and larvae as an alternative to oocyte cryopreservation have given more promising results (e.g. Asahina and Takahashi 1979, Olive and Wang 1997, Toledo and Kurorura 1990, Grout and McFadzen 1991, Lin et al. 1999, Wang et al. 2011, Paredes and Bellas 2014) but also new challenges. Both oocytes and embryos show high sensitivity to sub-zero temperatures, sensitivity to CPA exposure and lower permeability, embryos and larvae present some degree of organ cryoinjury or deformation of hard surfaces such as shells.

The fields of application of cryopreservation of marine organisms have extensively increased along the years, from the use in breeding industry, which is still one of the main applications (Adams et al. 2008, Robles et al. 2013, Suneja et al. 2014) with several patents have been filed within the last decade (James and Olive 2001, Dupré, Goldstein and Rojas 2012 or Tervit,

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Adams, Roberts, McGowan, Pugh, Smith and Janke 2006). Conservation of endangered species, DNA or germaplasm cryobanking of marine organisms additional applications (Odintsova et al. 2001, Hagedorn et al. 2006, Rhodes et al. 2006, Hagedorn et al. 2014, Odintsova and Boroda 2012, Paredes et al. 2013, Ohki et al. 2014). Cryopreservation of marine organisms/cells has also been applied to ecotoxicology, as embryo-larval bioassays are a very widespread and useful tool for marine water toxicity assessment (Grout and McFadzen 1991, McFadzen and Clearly 1994, Paredes and Bellas 2009, Bellas and Paredes 2011, Fabbrocini et al. 2012).

Summary of cryopreservation research on marine cells and organism; Oysters (Table 1) and other molluscs (Table 2), Cephalopods (Table 2), echinoderms (table 3), others (Table 4); from 1971 to 2017.

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Table 1.- List of cryopreservation studies published on Oysters sperm, oocytes, embryos and larvae from 1971 to 2017. Survival assessment results shown are the highest ones reported in each study. Cryoprotecting agents (CPAs) acronyms: Me2SO: Dimethyl sulfoxyde, EG: Ethylene glycol, PG: Propylene glycol, MetOH:Methanol, Gly:Glycine, G:Glycerol, AceT: Acetaminde, SUC: Sucrose, TRE:Trehalose, PVP: Polyvynyl pyrrolidone.

Species	Cell	Reference	Protocol	Survival Assessment
Crassostrea gigas	Sperm	Lanan (1971)	Me_2SO 20% (v/v).Hheld over LN2 vapor 2 min. Thawing rate not stated	10% Fertilization
		Hwang and Chen (1973)	Me_2SO 3.3-20% (v/v). Cooling by LN2 immersion. Thawing in water bath 20-24°C	79% Fertility
		Staeger (1974)	Me_2SO 20% + Gly 10-20% (v/v). Cooling rate 5 or 30 °C min^{-1}.Thawing in a water bath 22°C	36.3% Fertilization
		Kurokura *et al.* (1990)	Me_2SO 10% (v/v). Held 7 cm over LN2. Thawing at room temperature	30% Motility, 40% Survival, 57% Normal shape
		Yankson and Moyse (1991)	Me_2SO 10-20% + Gly 0.6% (v/v). Cooling rate 4.7 °C min^{-1}. Thawing in water bath 55°C 20 s.	89.7-93% Fertilization
		Bougrier and Rabenomanana (1996)	Me_2SO 10% (v/v). Held 5 cm. over LN2 vapor for 3 min. Thawing water bath 20°C 1 min.	Good motility, 92% Fertilization
		Smith *et al.* (2001)	Me_2SO 5% (v/v). Cooling rate 50°C min^{-1} . Thawing water bath 20°C 15 s.	80% Fertilization
		Gwo *et al.* (2003)	Me_2SO 10% (v/v). Cooling rate 15°C min^{-1}. Thawing in water bath 70°C 1 min.	40% Fertilization, Motility score 2 of 4
		Adams *et al.* (2004b)	Me_2SO 2.5-15% (v/v). Held 3 cm. over LN2 vapor for 10 min. Thawing in water bath 20°C for 5-8 min.	80% Fertilization
		Ieropoli *et al.* (2004)	EG 10% (v/v). Cooling rate 6°C min^{-1}. Thawing rate 74°C min^{-1}	60% Development to larvae
		Dong *et al.* (2005)	MetOH 6% (v/v). Two step cooling 4:45 °C min^{-1}. Thawing in water bath 40°C for 7 s.	70% Motility, 98% Fertilization
		Adams *et al.* (2008)	Me_2SO 5% (v/v) +TRE 0.54M. Held in a methanol bath -75°C for 10 min. Thawing in water bath 20°C for 5-8 min.	≥50% Fertilization at 10^7 sperm mL^{-1}
		Adams *et al.* (2011b)	Me_2SO 5% (v/v) + TRE 0.54M. Held in a methanol bath -75°C for 10 min. Thawing in water bath 20°C for 5-8 min.	≥80% Fertilization at 10^7 sperm mL^{-1}
		Paniagua-Chavez *et al.* (2011)	PG 15% (v/v). Cooling rate 2.5°C min^{-1}. Thawing rate 70°C min^{-1}	88% D-veliger larvae
	Oocytes	Chen *et al.* (1989)	Me_2SO 10% (v/v), Three step cooling rate 5:0.5:35 °C min^{-1}. Thawing in water bath 37°C	69, 51, 10% survival after 10 min.,1h., 10 days
		Naidenko *et al.* (1997)	Me_2SO 1M + TRE 0.15M +Antifreeze prot. 1.0 mg mL^{-1} +. Two step cooling 10:0.5 °C min^{-1}. Thawing in water bath 22-25°C	0.005 % D-veliger larvae
		Tervit *et al.* (2005)	EG 10% (v/v). Two step cooling 0.3:1 °C min^{-1}.Thawing in water bath 28°C	74.5% Fertilization, 30% D-veliger larvae, 28.4% Spat
		Salinas-Flores *et al.* (2008b)	EG 10% (v/v. Two step cooling 0.3:1 °C min^{-1}. Thawing water in bath 28°C	36% Fertilization
		Adams *et al.* (2011a)	EG 10%(v/v). Cooling rate 1°C min^{-1}. Thawing in water bath 28°C 5 s.	50% Development to D-veliger larvae
	Embryos	Renard (1991)	MetOH 0.5M + SUC 0.5M. Two step cooling 0.3:99.9 °C min^{-1}. Thawing in water bath 22-23 °C	0.1 % D-veliger larvae
		Lin *et al.* (1993b)	Me_2SO 1-2M. Two step cooling 1:2 °C min^{-1}. Thawing in water bath 28°C // Vitrification, combination of Me2SO, EG, PG	68% post-thaw rotatory motion, < 1% survival
		Chao *et al.* (1994a)	Me_2SO 2.5M + AceT 2.5M + TRE 0.15M. Optimum CPA combination regarding toxicity	67.7 % survival after 10h. post CPA exposure
		Gwo (1994)	PG 10% (v/v). Cooling rate 1.5 °C min^{-1}. Thawing in room temperature water	63% D-veliger larvae
		Naidenko *et al.* (1997)	Me_2SO 1M + TRE 0.15M + Antifreeze prot. 1.0 mg mL^{-1} . Two step cooling 10:0.5 °C min^{-1} .Thawing in water bath 22-25°C	0.001% D-veliger larvae
		Lin *et al.* (1999)	Me_2SO 2M or G 2M. Two step cooling 1:2 °C min^{-1}. Thawing in water bath 28 °C	83% Rotatory movement 1-2h. post thawing
		Lin and Chao (2011)	Me_2SO 2M. Two step cooling rate 1:2 °C min^{-1}. Thawing in water bath 28°C	78% Survival
	Larvae	Grout and McFadzen (1991)	Me_2SO 15% (v/v). Two step cooling 12/17:40/50 °C min^{-1}. Thawing rate 5/15°Cmin^{-1}	70-90% Survival after 10 days post thawing
		Gwo (1994)	PG 10% (v/v). Cooling rate 1.5 °C min^{-1}. Thawing in water bath at room temperature	37.8% D-veliger larvae
		Chao *et al.* (1997)	Me_2SO 3M + TRE 0.09M. Two step cooling 1:2°C min^{-1}. Thawing in water bath 28°C	75% post thaw larval movement
		Naidenko *et al.* (1997)	Me_2SO 1M + TRE 0.15M +Antifreeze prot. 1.0 mg mL^{-1}. Two step cooling 10:0.5 °C min^{-1}. Thawing in water bath 22-25°C	0.001% D-veliger larvae
		Usuki *et al.* (2002)	Me_2SO 1.5M + TRE 250 mM. Cooling rate 1.5°C min^{-1}. Thawing in water bath 26°C	≥90% motility post-thaw, 10% Normal shell by 7 d.

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		Lin and Chao (2011)	Me_2SO 2M. Two step cooling 10:0.5 °C min^{-1}. Thawing in water bath 22-25 °C	78% D-veliger rotatory motion
		Paniagua-Chavez *et al.* (2011)	PG 15% (v/v). Cooling rate 2.5°C min^{-1}.Thawing rate 70°C min^{-1}	24% D-veliger larvae
		Paredes *et al.* (2013)	EG 10% (v/v). Two step cooling 1:0.5 °C min-1. Thawing in water bath 28°C 5 sec.	60% D-veliger larvae
		Suneja *et al.* (2014)	EG 10% (v/v) +TRE 0.4M.Cooling rate 1°C min^{-1}. Thawing in water bath 28°C	≤5% survival after 22 days
		Suquet *et al.* (2014)	EG 10% (v/v) + PVP 1% (w/v). Two step cooling rate 1:0.3°C min^{-1}. Thawing in water bath 37°C 10 s.	20-50% Survival/70% motility of 2nd generation
Ostrea edulis	sperm	Vitiello *et al.* (2011)	EG 15% (v/v). Cooling rate 3°C min^{-1}. Thawing rate 60°C min^{-1}	Motility of sperm in the highest class
		Horváh *et al.* (2012)	Me_2SO 10% (v/v). Held over LN2 vapor 3 min. Thawing in water bath 40°C 13 s.	8% Motility
Ostrea angasi	Sperm	Hassan et al. (2017)	Me2SO 10% + TRE 0.45M. 30 min exposure. Cooling rate -3°C/min from 4 to -80°C. Thawed at 40°C for 8 s.	44.4% sperm motility, 49.2% plasma membrane integrity
		Hassan et al. (2017)	EG 15% + TRE 0.2M. 20 min of exposure. Straws of 0.25mL, 8 cm above LN surface for 10 min and then plunged into LN. Thawing at 40°C water for 6s.	66.2 sperm motility, 67.3& plasma membrane integrity
Saccrostrea glomerata	Larvae	Liu and Li (2008)	Me_2SO 10% (v/v). Three step cooling 7:1:2°C min^{-1}. Thawing rate 1356-904°C min^{-1}	45-90% Rotary motion, Cilia movements 96 h.
Crassostrea angulate	Sperm	Riesco et al. (2017)	Me2SO 10%. Samples in straws. Faster cooling rate: -6°C/min from 0 to70°C. Samples plunged directly into LN.	80% motility/viability
Crassostrea iredalei	Sperm	Yankson and Moyse (1991)	Me_2SO 10-15% (v/v) + Gly 0.6% (v/v). Cooling rate 4.7°C min^{-1}. Thawing in water bath 55°C 20 s.	30.1-36.4% Fertilization
Crassostrea rhizophorae	Sperm	Sansone *et al.* (2005)	Me_2SO, PG,EG less toxic in ranges 15-18%	No abnormalities after 24h. exposure
	Oocytes	Sansone *et al.* (2005)	Me_2SO, PG,EG less toxic in ranges 12-19%	No abnormalities after 24h. exposure
	Larvae	Sansone *et al.* (2005)	MetOH, Me_2SO 55%	No abnormalities after 24h. exposure
Crassostrea tulipa	Sperm	Yankson and Moyse (1991)	Me_2SO 15-20% (v/v) + Gly 0.6% (w/v). Cooling rate 4.7°C min^{-1}. Thawing in water bath 55°C 20 s.	71% Fertilization
Crassostrea virgnica	Sperm	Hughes (1973)	Me_2SO 10% (v/v). Two step cooling rate 1:5.5°C min^{-1}. Thawing at air 21°C	2% Fertilization
		Zell et al. (1979)	Me_2SO 8% (v/v) + Gly 80mM. Variable cooling rate 5-13°C min^{-1}. Thawing in water bath 60°C 10s.	91% Fertilization, Normal development 11 days
		Paniagua-Chavez *et al.* (1998b)	PG 10% (v/v) + SUC 0.25M. Cooling rate 2.5°C min^{-1}. Thawing in water bath 70°C 15 s.	1.66% Survival 12h. post fertilization
		Paniagua-Chavez *et al.* (2001)	PG 10% (v/v) + SUC 0.25M.Cooling rate 2.5°C min^{-1}. Thawing in water bath 70°C 15 s.	57% Trochophore larvae
		Yang *et al.* (2012)	Me_2SO 20% (v/v). Cooling rate 25°C min^{-1}. Thawing in water bath 40°C 7-8s.	20% Fertilization
		Paniagua-Chavez *et al.* (1998a,2011)	PG 15% (v/v). Cooling rate 2.5°C min^{-1}. Thawing in water bath 70°C 15 s.	88% Larvae
	Larvae	Paniagua chavez *et al.* (2001)	PG 10% (v/v) + SUC 0.25M. Cooling rate 2.5°C min^{-1}. Thawing in water bath 70°C 15 s.	100% Motility of trochophore larvae
		Paniagua-Chavez *et al.* (2011)	PG 15% (v/v). Cooling rate 2.5°C min^{-1}. Thawing in water bath 70°C 15 s	24% Survival
Saccrostrea cucullata	Sperm	Yankson and Moyse (1991)	Me_2SO 10-15% (v/v) + Gly 0.6% (w/v). Cooling rate 4.7°C min^{-1}. Thawing in water bath 55°C 20 s.	78% Fertilization
Pinctada fucata martensii	Sperm	Kawamoto *et al.* (2007)	MetOH 10% (v/v). Cooling rate 19°C min^{-1}. Thawing rate 800°C min^{-1}	63% Sperm motility
	Larvae	Choi and Chang (2003)	Me_2SO 2M + SUC 0.2M. Cooling rate 1°C min^{-1}. Thawing in water bath 20°C 10 s.	91% Rotatory motion 1h. post thawing
Pinctada margaritifera	Sperm	Lyons *et al*. (2005)	Me_2SO 5% + TRE 1M. Held over LN2 for 15 min. Thawing in water bath 25°C 30 s.	89% Sperm motility

Table 2.- List of cryopreservation studies published on other mollusks (clams, mussels, abalone) and cephalopods sperm, oocytes, embryos and larvae from 1971 to 2017. Survival assessment results shown are the highest ones reported in each study. Cryoprotecting agents (CPAs) acronyms: Me2SO: Dimethyl sulfoxide, EG: Ethylene glycol, PG: Propylene glycol, MetOH:Methanol, Gly:Glycine, G:Glycerol, AceT: Acetaminde ,SUC: Sucrose, Glu: Glucose, TRE:Trehalose, PVP: Polyvynyl pyrrolidone.

Species	Cell	Reference	Protocol	Survival Assessment
Meretrix lusoria	Embryos	Lin *et al.* (1994)	Me_2SO 2M. Two step cooling rate 1:2 °C min^{-1}. Thawing in water bath 28°C	72% Motility post thawing
		Chao *et al*. (1997)	Me_2SO 2M +TRE 0.06M. Two step cooling rate 1:2 °C min^{-1}. Thawing in water bath 28°C	83% Motility post thawing
		Lin and Chao (2011)	Me_2SO 2M. Two step cooling rate 1:2 °C min^{-1}. Thawing in water bath 28°C	72% Survival
Ruditappes philipparum	Larvae	Renard *et al.* (1989)	MetOH < EG < PG <Me2SO < G Increasing toxicity	Toxicity tests
	Primary	Odintsova et al. (2001)	Me2SO	Trypan blue

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Mizuchopecten yessoensis	Sperm	Yang et al. (2007)	Me2SO 16 %. Freezing method NPM. Freezing 20 cm above LN for 3 min; 3 cm above LN for 10 min. Thawing temperature ℃.	45% Motility, 26% Fertilization (500–600:1)
Messodema donacium	Sperm	Joo *et al*. (2002)	Me_2SO 1M toxicity test	44% Motility after exposure
		Dupré and Guerrero (2006,2011)	Me_2SO 2M. Cooling rate 20°C min-1. Thawing rate 72°C min^{-1}	97% Fertility
Spinula sachaliniensis	Larvae	Choi *et al.* (2008)	EG 2M + SUC 0.2M. Cooling rate 1°C min^{-1}. Thawing in water bath 25°C 10 min.	96% Survival
Perna canalinulus	Sperm	Adams *et al.* (2011b)	Me_2SO 12% (v/v)+ TRE 0.25M. Cooling rate 5°C min^{-1}. Thawing in water bath 28°C	60% Fertilization rate
	Oocytes	Adams *et al.* (2009)	EG 10% (v/v) + TRE 0.4M. Two step cooling ramp 1:0.5°C min^{-1}. Thawing in water bath 28°C	1% D-yield larvae
	Larvae	Paredes *et al.* (2012)	EG 10% (v/v) + TRE 0.2M. Cooling rate 1°C min^{-1}. Thawing in water bath 28°C 6 s.	Good motility, 60% fertilization
		Rusk (2012)	EG 20% (v/v) Two step cooling ramp 1:0.5°C min^{-1}. Thawing in water bath 28°C	Survival 18 day larval rearing between 0.03-0.5%
Mytilus galloprovinciallis	Sperm	Di Mateo *et al.* (2009)	EG 7% (v/v). Cooling rate 1°C min^{-1}. Warming rate 60°C min^{-1}	90% Fertilization rate
		Liu et al. (2015)	Me2SO 8% + GLY 0.8%. Non-programmable freezing technique. Sperm cryopreserved at 7.8cm above the LN surface. Thawed in a 60℃ SW bath.	95% Fertilization rate
	Oocytes	Liu and Li (2015)	7.5% FIC and 10% EG, post-thaw CPA removal medium 9% sucrose.	14% D-larval rate was determined at 40 – 48 h
	Larvae	Wang *et al.* (2011)	Me_2SO 5% (v/v). Two step cooling rate 1.5:0.4°C min^{-1}. Warming rate 2664°C min^{-1}	85% Moving cilia, 55% swimming motion, 8% Food intake after 21
		Paredes *et al.* (2013)	EG 10% (v/v)+ TRE 0.2M. Cooling rate 1°C min^{-1}. Thawing in water bath 28°C 6 s.	50 % D-veliger stage
Mytilus edulis	Embryo	Toledo *et al.* (1989)	Me_2SO 1.5M. Two step cooling rate 5:0.5°C min^{-1}. Thawing in water bath 18°C	48.8% survival
Mytilus trossulus	Embryo	Kostetsky *et al.* (2007)	Me_2SO 10% (v/v) + TRE 1.5% (w/v) + Vitamins + lipids. Cooling rate 7°C min^{-1}. Thawing in water bath 20-22°C	35% Viable cells
	Larvae	Odintsova *et al.* (2009)	Me_2SO 6% (v/v) + 40mM TRE + 0.15% (w/v) Antioxidants. Cooling rate 7°C min^{-1}. Thawing in water bath 10-15°C	40% Survival
	Somatic	Odintsova et al. (2006)	Me2SO 10% + TRE 3-30 mg/ml by two-step freezing.	60-95% cell viability by RNA activity.
Haliotis rufescens	Sperm	Salinas-Flores *et al.* (2005)	G 10% (v/v). Cooling rate 16°C min^{-1}. Thawing in water bath 45°C 8 s.	48% Motility, 56% Intact membrane ,29% Fertilization rate
Haliotis laevigata	Sperm	Liu *et al*. (2014)	Me_2SO 6% (v/v) + Glu 1% (w/v). Held 5.2 cm. over LN2 vapor 10 min. Thawing in water bath 18°C	84% Fertilization rate
		Zhu et al. (2014)	Me2SO 6% (v/v) + Glu 1% (w/v). Held 5.2 cm. over LN2 vapor 10 min. Thawing in water bath 18 LC	80% Fertilization rate
		Lui et al (2016)	Me2SO 10%. + GLY 0.6% + TAURINE 0.2% + 0.02 L-ascorbic acid. Cooling rate -5℃/min to -30℃ from 0℃. Thawing at 40℃ and 18℃ SW.	90% Fertilization rate, 20% Post-thaw sperm motility
Haliotis diversicolor	Sperm	Gwo et al. ()	Me2SO 10%. Cooling rate of -15 C/min to -120℃-	50% Fertilization rate
	Oocytes	Tsai and Chao (1994), Chao and Tsai 1994b	Me_2SO 8% (v/v)+ Glu 5% (w/v), Held over LN2. Thawing at room temperature	48-92% Hatching
		Yang *et al.* (2013)	Me_2SO 2M. Cooling rate 1.5°C min^{-1}. Thawing rate 112°C min-1	48.8% Osmotic active, 23.7% Hatching
	Embryos	Lin and Chao (2011)	Me_2SO 2M + G 2M. Vitrification	14% Survival
Haliotis midae	Embryos	Roux *et al.* (2008)	Toxicity study PG < MetOH < Me2SO <Gly	Best survivals with 5-15% PE
Haliotis iris	Sperm	Adams *et al.* (2011b)	Sucrose 1.6M. Cooling rate 5°C min^{-1}. Thawing in water bath 14-18°C 40 s.	80% Fertilization rate
H. discus hanai	Sperm	Khang et al (2004)	5 % GLY 1:9. Cooling rate -50℃/min to -80℃.	71.4% sperm survival rate
Haliotis tuberculata	Somatic	Poncet et al. (2002)	Centrifugation at 4℃, 10 min. Me2SO 10% (v/v) or Gly 10% (v/v), then 12h at -80℃ .Thawing in water bath at 25℃.	Trypan blue exclusion tests, 76% Me2SO , 68% gly
	Haemocy -tes	Poncet and Lebel (2003)	Gly 20%. From 23℃, Cooling rate -1℃*min^{-1} to -5℃. Isotermal holding 0,6 min.	83% Cell viability assessed by DNA , 68% Cell viability by proteomic, 73%Metabolic
Illex coindetii	Spermato -phores	Robles *et al.* (2013)	Me_2SO 15% (v/v). Held 1 cm over LN2 30 min, Thawing in water bath 30°C for 2 min. 20 s.	5-10% motile sperm, 5% membrane integrity, 9.4% sperm with active mitochondria, 85% sperm with damaged membranes

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Table 3.- List of cryopreservation studies published on echinoderms (sea urchins, sand dollars, starfish, sea cucumbers) sperm, oocytes, embryos and larvae from 1971 to 2014. Survival assessment results shown are the highest ones reported in each study. Cryoprotecting agents (CPAs) acronyms: Me2SO: Dimethyl sulfoxide, EG: Ethylene glycol, PG: Propylene glycol, MetOH:Methanol, Gly:Glycine, G:Glycerol, AceT: Acetaminde , SUC: Sucrose, Glu: Glucose, TRE:Trehalose, PVP: Polyvynyl pyrrolidone. * Only partial abstracts were available, information completed from bibliographical references.

Species	Cell	Reference	Protocol	Survival Assessment
Hemicentrotus pulcherrimus	Embryos	Asahina and Takahashi (1978, 1979)	Me_2SO 1.5M. Cooling rates 10-40°C min^{-1}. Thawing in air 15°C min^{-1}	10% Development to larvae
	Larvae	Asahina and Takahashi (1977)	Me_2SO 1M. Cooling rates 10-40°C min^{-1}. Thawing in air 15°C min^{-1}	90% Survival
Strongylocentrotus nudus	Larvae	Asahina and Takahashi (1979)	EG 1.5M. Cooling rate 10°C min^{-1}.Thawing rate 7°C min^{-1}	90% Active swimming
Strongylocentrotus intermedius	Embryo	Asahina and Takahashi (1978, 1979)	EG 1.5M. Cooling rate 10°C min^{-1}. Thawing rate 7°C min^{-1}	90% Active swimming
		Gakhova *et al.* (1988)	Me_2SO 1-1.5M. Cooling rate 6-8°C min^{-1}. Thawing in water bath 19°C	≥90% survival
		Naidenko *et al*. (1991)	Me_2SO 1.5M. According to Gakhova et al. (1988)*	0.1-0.2% Development to 2nd generation
		Naidenko *et al.* (1998)	Me_2SO 1M + 1 mg ml^{-1} Antioxidant. According to Gakhova et al. (1988)*	60% swimming post-thaw, 1% develop.
		Odintsova *et al.* (2009)	Me_2SO 6% (v/v)+ 40 mM TRE + 0.15% (w/v) Antiox. Cooling rate 7°C min^{-1}. Thaw in water bath 10-15°C	40% survival
	Larvae	Asahina and Takahashi (1979)	EG 1.5M. Cooling rate 10°C min^{-1}. Thawing rate 7°C min^{-1}	90% Development
		Naidenko *et al.* (1998)	Me_2SO 1M + 1 mg ml^{-1} Antiox., According to Gakhova et al. (1988)*	20% Active Swimming
Anthocidaris crassispina	Sperm	Wu *et al.* (1990)	According to H. Kurokura et al. (1989)*	10% Motility
Loxechinus albus	Larvae	Barros *et al.* (1996)	Me_2SO 1M. Two step cooling rate 3:10 °C min^{-1}. Thawing in water bath 15°C 30 s.	77% Survival after 24 h., 55% Survival after 21 d.
Tetrapigus niger	Sperm	Barros *et al.* (1996, 1997)	Me_2SO 1.2M. Two step cooling rate 6:25 °C min^{-1}. Thawing in water bath 17°C 12 s.	96% Fertilization, 56% Development after 24h.
Strongylocentrotus droebachiensis	Sperm	Dunn and McLachlan (1973)	Me_2SO 12% (v/v).Cooling rate 5°C min^{-1}. Thawing at room temperature 45 min.	Motility score 4 of 10
Evechinus chloroticus	Sperm	Adams *et al.* (2004a)	Me_2SO 2.5-7.5% (v/v). Cooling rate 50°C min^{-1}. Thawing in water bath 15°C 30 sec.	95% Fertilization
	Larvae	Adams *et al.* (2006)	Me_2SO 1.5M. Cooling rate 2.5°C min^{-1}. Thawing in water bath 15°C 30 s.	91% Motility
Paracentrotus lividus	Sperm	Fabbrocinni *et al.* (2014)	Me_2SO 7% (v/v). Cooling rate 20°C min^{-1}. Thawing rate 15°C min^{-1}	90% motility, 50% Normal Larvae
	Oocytes	Paredes and Bellas (2009)	Me_2SO NOEC:0.5M, EG NOEC:1M, PG NOEC:0.68M	Toxicity tests of CPAs
	Embryos	Bellas and Paredes (2011), Paredes and Bellas (2014)	Me_2SO 1.5M + 0.04M TRE. 1°C min^{-1}. Thawing in water bath 18°C	50-80% Normal larvae after 96 h.
Urechis unicintus	Sperm	Khang *et al.* (2004a)	Me_2SO 15% (v/v). Cooling rate 30°C min^{-1}. Thawing in water bath 30°C	41% Motility
Echinometra lucunter	Oocytes/Embryos	Ribeiro et al. (2017)	Tocicity tests of CPAs	Less toxic for oocytes Me2SO, Blastulas EG
Pseudocentrotus depressus	Sperm	Kurokura *et al.* (1989)	Me_2SO 10% (v/v). Cooling rate 6°C min^{-1}. Thawing rate 10-15°C min-1	13-33% Fertilization
Echinarachnius parma	Sperm	Dunn and McLachlan (1973)	Me_2SO 15% (v/v). Cooling rate 5°C min^{-1}. Thawing at room temperature 45 min.	Motility score 4 of 10
Asterias vulgaris	Sperm	Dunn and McLachlan (1973)	Me_2SO 7.5-40% (v/v). Cooling rate 5°C min^{-1}. Thawing at room temperature 45 min.	Motility score 3 of 10
Asterina minuata	Larvae	Köseglu *et al.* (2001)	Me_2SO 1.5M. Cooling rate 1°C min^{-1}. No thawing	77% IIF
Apostichopus japonius	Sperm	Shao *et al.* (2006)	Me_2SO 15% (v/v). Held 6 cm. over LN2. Thawing in water bath 37°C	76% motility

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Table 4.- List of cryopreservation studies published on cnidaria (corals, anemones), Annelida (sandworm), Arthrophods (barnacles) and Rotifera cells, sperm, embryos and larvae from 1971 to 2017. Survival assessment results shown are the highest ones reported in each Cryoprotecting agents (CPAs) acronyms: Me2SO: Dimethyl sulfoxide, EG: Ethylene glycol, PG: Propylene

Species	Cell type	Reference	Protocol	Survival Assessment
Pocillipora damicomic	Fragments	Hagedorn *et al.* (2013)	Me_2SO 1M. Chilling sensitivity experiments	75% intact tissue, zooxantelles declined after 5 minutes of chilling
		Feuillassier *et al.* (2014)	EG > MetOH >Me2SO>Glycerol Increasing toxicity	Tolerate 2.45M CPA combination of SUC+Me2SO+EG+MetOH
Acropora difitifera	Sperm	Ohki *et al.* (2014)	MetOH 20% (v/v) + Suc 0.9M. Held 4 cm over LN2 4 minutes (Cooling rate 40°C min^{-1}). Thawing not stated	63% Fertilization rate
Acropora millepora	Pluripotent cells	Hagedorn *et al.* (2012)	Me_2SO 10% (v/v) +BSA 1% (w/v). Cooling rate 0.5°C min^{-1}. Thawing in water bath 30°C	90% Intact
Acropora tenuis	Sperm	Hagedorn *et al.* (2012)	Me_2S0 10% (v/v). Cooling rate 18°C min^{-1}. Thawing in water bath 30°C	35% development to larvae after 12 h.
	Pluripotent cells	Hagedorn *et al.* (2012)	Me_2SO 10% (v/v) +BSA 1% (w/v). Cooling rate 0.5°C min^{-1}. Thawing in water bath 30°C	42% Intact
Junceella juncea	Sperm	Tsai *et al.* (2011)	Me_2SO NOEC:3M, MetOH NOEC:3M, PG NOEC:1M, EG NOEC:2M, Glycerol NOEC:1M	Toxicity tests of CPAs
Junceella fragilis	sperm	Tsai *et al.* (2011)	Me_2SO NOEC:3M, MetOH NOEC:2M, PG NOEC:1M, EG NOEC:2M, Glycerol NOEC:2M	Toxicity tests of CPAs
Ciona intestinalis	Sperm	Sorrenti *et al.* (2014)	Me_2SO 10% (v/v). Two step cooling rate 1:13°C min^{-1}. Thawing in water bath 30°C 10s.	Sperm motility class 3 of 5
Nereis virens	Larvae	Olive and Wang (1997)	Me_2SO 1.4M. Cooling rate 2.5°C min^{-1}. Thawing in a water bath 20°C	86% Survival
Balanus amphitrite	Larvae	Khin-Maung-Oo *et al.* (1998)	Me_2SO 1.5M. Two step cooling rate 0.5°C min^{-1} (until seeding temperature) followed by LN2 plunging. Thawing in water bath 50°C 10 s.	78% Larvae recovered, 19.8% metamorphose to cyprid
Brachionus plicatilis	Embryos	King *et al.* (1983)	Me_2SO 6% (v/v). Cooling rate between 1-2°C min^{-1}. Thawing in water bath 37°C	2% Survival
		Toledo and Kurokura (1998)	Me_2SO 10% (v/v). Two step cooling rate 5:0.3°C min^{-1}. Thawing in water bath 19-20°C	55% Survival after 30 days, survivors laid eggs

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4.2 Marine Micro and macroalgae cryopreservation

Over the past 45 years' cryopreservation has been employed by algal Biological Resource Centres (BRCs) to conserve their holdings of microalgae (Morris 1976; Watanabe et al. 1992; Bodas *et al*. 1995; Wood et al. 2008). Although today well in excess of the >3000 strains conserved in the COBRA project (Day et al. 2005) are held by collections worldwide in a cryopreserved state; however, many taxa remain recalcitrant to conventional cryopreservation methodologies.

For many non-gas-vacuolated cyanobacteria robust methodologies have been developed (Table 5). There remain some constraints on the materials that can be successfully held. However, new cryoprotective approaches have considerably extended the range and diversity of taxa and in some cases the levels of post-thaw viability, of cryopreserved cyanobacterial. On-going challenges include the preservation of more structurally complex taxa, those with large amounts of mucilage, many gas vacuolated strains and non-axenic isolates where the levels of commensal bacteria and/or fungi are relatively high.

As with other biological materials, the use of ultra-low temperatures permits the storage of living microalgae in a state of "suspended animation" for considerable periods with no significant reduction in viability on up to 22 years of cryostorage of a range of algae having been observed (Day et al., 1997) and as yet unpublished data indicates that if the storage temperature is stable samples held for up to 40 years have undiminished levels of viability (Day et al, in prep). Furthermore, there is a slowly expanding evidence-base that algae retain the capacity of cells recovered from cryostorage to produce the metabolite(s) of biotechnological interest, e.g., Hédoin et al. (2006) and Hipkin et al. (2014).

There is an extensive literature on methodological development and application for conservation of microalgae. Most approaches have employed conventional colligative cryopreservation using either passive coolers or controlled rate systems. Most procedures can be categorized as two-step freezing protocols. These two-step protocols require the addition of a cell-permeating Cryoprotective Agent/ Cryoprotectant (CPA) to an algal culture prior to its freezing, and then the culture is cooled at a controlled rate to some sub-zero temperature (Step 1). Next, the sample is cooled rapidly to the final storage temperature (Step 2). The culture can be maintained at the storage temperature for an indefinite period of time.

Many variations have been employed within this broad framework. Parameters that may affect the viability of a cryopreserved algal culture include: the growth stage or physiological condition of the culture at the time of its harvesting for cryopreservation; the selected CPA, its concentration, and the temperature at which it is added; the cooling protocol, i.e., how fast it is cooled at each stage of the cooling process and the precision with which the cooling regime is controlled; the intermediate temperature at which Step 1 is terminated and the culture is rapidly cooled to the final storage temperature; the thawing conditions; and the incubation conditions of the culture during its recovery.

Cryoprotective agents generally must be added at high concentrations to afford protection from cell damage during freezing and thawing. Although two classes of CPAs may be distinguished: (1) agents that passively move through the plasma membrane to equilibrate between the extracellular solution and the cell interior (penetrating or permeating CPAs), and (2) those that

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do not pass through the plasma membrane and remain in the extracellular solution (non-penetrating or non-permeating CPAs). With the exception of a handful of publications e.g., Gäbler-Schwarz et al. (2013) penetrating CPAs are the norm in microalgal cryopreservation. Three penetrating CPAs have been utilized quite extensively for algal cryopreservation: methanol (MeOH), dimethylsulphoxide (DMSO; = Me_2SO), and glycerol (Taylor and Fletcher 1998). Many marine microalgae are most effectively cryopreserved with DMSO (Day and Brand, 2005), while glycerol is effective for *Tetraselmis* (Day and Fenwick 1993). Ethylene glycol and formamide (rarely used as algal CPAs), as well as DMSO, may decrease the cell membrane permeability for ions and lower the membrane potential (Chekurova et al. 1990). Penetrating cryoprotectants may also act as free radical scavengers (Benson 1990). The relative rates of transport of different CPAs with respect to water greatly affect the direction, extent, and rate of transient changes in cell volume. The difference in transport rates of DMSO and MeOH may help explain why many freshwater and terrestrial algae with robust cell walls that can tolerate transient swelling are best cryopreserved with methanol, while many marine species, which often lack a strong wall, are more effectively cryopreserved with DMSO (Day and Brand, 2005).

Penetrating CPAs are toxic at high concentrations. Prolonged exposure to methanol at concentrations that are used for cryoprotection (typically 5-%–10% v/v) is toxic to *Euglena gracilis*, and even short- term (20 min) exposure to concentrations greater than 15% (v/v) may be damaging (Fleck 1998). Monohydric alcohols, DMSO, and ethylene glycol denature enzymes at room temperature, and DMSO destabilizes proteins (Adam et al. 1995). However, DMSO may protect isolated enzymes during freezing (Adam et al. 1995, Anchordoguy et al. 1992). This apparent paradox has been attributed to temperature-dependent, hydrophobic interactions between DMSO and non-polar moieties of proteins. At temperatures below –-22^{o}C, low concentrations of rapidly permeating cryoprotectants may act as cryosensitizsers, thereby accelerating membrane damage (Santarius 1996). In addition, DMSO has been observed to cause artificial phospholipid bilayers to become leaky due to a hydrophobic association between DMSO and the bilayer (Anchordoguy et al. 1992). Thus, a permeating CPA should be added to a culture only immediately prior to its cryopreservation and should be removed as soon as possible after thawing, or at least diluted down to a level where damage is unlikely to occur.

Occasionally, permeating CPAs are added after cultures are cooled to 0°C or lower, in order to minimize intracellular toxicity (Fleck 1998). However, the CPA should not be added after ice has formed in the culture. Due to slow CPA membrane transport at low temperatures, an algal culture to which CPA has been added at a low temperature should be incubated several minutes before it is further cooled, in order to ensure adequate equilibration of the CPA and water across the plasma membrane. An equilibration time as long as 30 minutes may be required if DMSO is added at 0°C or lower, while less equilibration time is required for MeOH. Concentrations of MeOH or DMSO less than 2% (v/v) are seldom effective as CPAs, while concentrations higher than 12% (v/v) are often toxic (Day and Brand, 2005). Within this range the most effective concentration varies greatly among species, sometimes even among closely related strains. Many strains of microalgae in the UTEX and CCAP collections have been successfully cryopreserved utilizing with 5% (v/v) MeOH or 5% -to 8 % (v/v) DMSO, but the most effective concentrations for individual strains often must be determined empirically. For example, concentrations of DMSO higher than 9% (v/v) are toxic to *Pfiesteria*, while

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concentrations lower than 5% are ineffective in preventing freezing/thawing damage (Day and Brand, 2005).

Passive freezing systems are often employed in two-step freezing protocols. In Step 1, cryogenic vials containing algal cultures prepared for cryopreservation are placed into a static system (an insulated container) that is exposed to a very low temperature (typically by its insertion into a -80°C freezer). The insulation of the container retards heat transfer, so the contents of the container cool relatively slowly. The interior of a properly designed passive freezing system cools at an approximately linear rate from 0°C to less than –40°C. When the contents of the cryogenic vials reach a sufficiently low temperature (typically –30 to –80°C), they are removed from the insulated container and transferred directly to a permanent ultra-cold storage location such as a liquid nitrogen dewar (Step 2 of two-step cooling protocols). Step 1 cooling is terminated at –45°C to –55°C when MeOH is used as the CPA at UTEX, and at –30°C to –40°C when DMSO is used as the CPA at the CCAP. The sample must be transferred quickly from the insulated container to a permanent storage location to avoid excessive warming during the transfer time. The temperatures of the transferred samples quickly (within a few minutes) approach the internal temperature of the storage container (–-196^{o}C for liquid nitrogen). Commercially available passive coolers including “Mr. Frosty” (Nalgen Nunc International, Rochester, NY, USA) and “Handi-Freeze” (Taylor Wharton, Nottingham, UK), are inexpensive and provide highly reproducible cooling rates. The chamber cools at approximately -1°C /min to -50°C. A convenient protocol suitable for many strains of microalgae is to (1) pre-chill the freezing canister to 4°C, (2) place room-temperature cryogenic vials containing algal cultures to be cryopreserved into the pre-chilled canister, (3) immediately place the closed canister into a -80°C freezer, and (4) remove the canister after the desired temperature is reached and quickly transfer the frozen vials to an ultra-cold storage vessel.

Some algae require a more carefully controlled cooling rate or a more complex cooling pattern than can be achieved with passive freezing devices. A variety of commercial instruments (e.g., Biotronics, Leominster, UK; Planer Products, Sunbury, UK; CryomMed, Thermo Electron Corporation, Medford, Massachusetts USA; Gordinier Electronics, Roseville, Michigan; I, USA; CryoLogic, Musgrave, Victoria, Australia) allow accurate control and manipulation of the cooling regime. A temperature probe is inserted into a cryogenic vial that contains solution very much like the contents of vials of cultures prepared for cryopreservation. The vials prepared for cryopreservation, along with the vial containing the temperature probe, are then inserted into the cooling chamber of the controlled-cooling-rate freezer. This probe, along with an additional probe projecting into the cooling chamber, is connected to an electronic device that regulates the entry of vapour-phase nitrogen into the chamber. The rate of cooling is determined by the rate of entry of cold nitrogen into the chamber in response to temperatures sensed by the probes according to a user-defined cooling protocol. Electronic and printed outputs that describe the cooling protocol allow the details of each cryopreservation run to be recorded automatically. These controlled-cooling-rate freezers can be programmed to produce a wide range of customized cooling protocols. The most commonly employed protocol at the CCAP involves cooling at 1^{o}C/ min from +20^{o}C to -40^{o}C, then holding the sample at -40^{o}C for 30 minutes prior to transferring cryogenic vials to liquid nitrogen (Day and Brand 2005). A more complex cooling program successfully employed for marine strains involves cooling the contents of cryogenic vials from ambient temperature to 4°C at -1°C/min, then holding the temperature constant for up to 5 minutes. This is sometimes required for adequate penetration

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of the CPA, the vials are next cooled at -1°C/min until they reach -9°C. Seawater remains a supercooled liquid at that temperature. The cooling chamber is then cooled rapidly to -45°C to induce ice nucleation and rapidly removes the latent heat of fusion. The vials are then cooled at -1°C/min until they reach -45°C, which is below the eutectic. The vials are then rapidly finally transferred from the cooling chamber to a liquid nitrogen storage system (Day and Brand, 2005).

Additionally, encapsulation-vitrification and encapsulation two-step cooling based methods have been developed (Figure 1). These approaches have been successfully trialled for a range of, mostly freshwater, organisms and validated by three algal BRCs (Elster et al., 2008; Harding et al., 2008; Lukešová et al., 2008). However, because of the procedural complexity this approach has not as yet been more widely adopted for routine use in any algal collection.

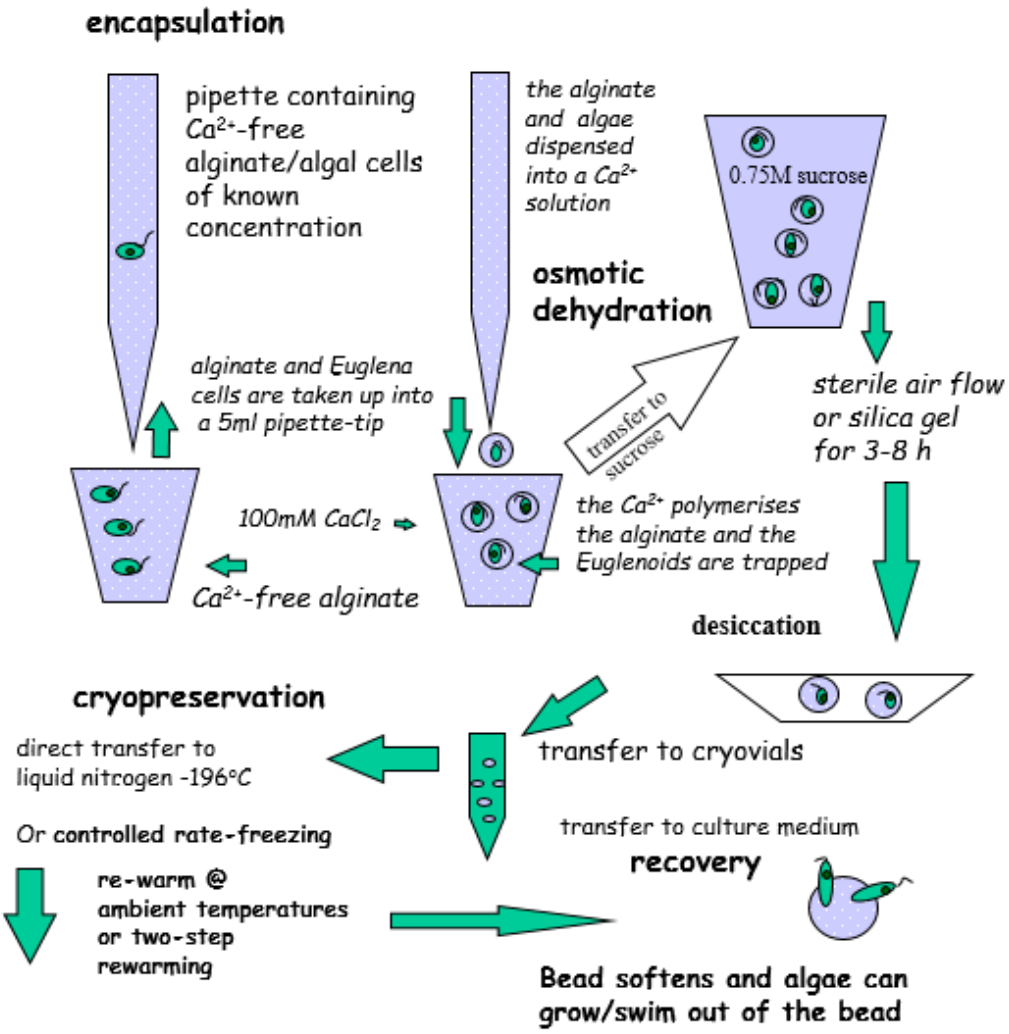

Figure 1. Encapsulation-vitrification and encapsulation-2 step cooling methods applied to microalgae (Adapted from Day and Brand, 2005)

Although there are increasing interests in the exploitation of macroalgae and the need to conserve materials, to date, relatively very few macroalgae have been held by collections in a cryopreserved state (Heesch et al. 2012), although there is considerable potential to do so (Table 6 summarises exemplar methodologies that have been successfully applied).

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Table 5. Standard cryopreservation protocols used for cyanobacteria

Origin of strains studied	Cryoprotectant	Cooling protocol		Reference
Freshwater	DMSO 5% (v/v)	(1 step) Direct storage at -80^{o}C		Romo and Becares (1992)
Freshwater	DMSO 5% (w/v)	(2 step) -30^{o}C for 15 min*	Liquid N_2	Morris (1978)
Marine/hypersaline	DMSO 10% (w/v)	(2 step) -30^{o}C for 15 min*	Liquid N_2	Morris et al. (unpublished)
All	DMSO 10% (v/v)	(2 step) -1^{o}C min^{-1} to -30^{o}C	Liquid N_2	Watanabe et al. (1992)
All	DMSO 10% (v/v)	(2 step) -1^{o}C min^{-1**} to -40^{o}C***	Liquid N_2	Day and Brand (2005)
Terrestrial/ freshwater****	DMSO 8% (w/v)	(2 step) -1^{o}C min^{-1**} to -40^{o}C***	Liquid N_2	Bodas et al. (1995)
Freshwater	Encapsulation in 1.2% alginate	Desiccation in 0.5M sucrose	Liquid N_2	Hirata et al. (1996)

DMSO - Dimethyl sulphoxide
*Immersion of cryovial in a pre-cooled alcohol bath (see text for detail)
**Achievable using either a passive -freezer unit placed in a -80^{o}C Freezer, or a proprietary controlled rate cooler (see text for detail)
***Terminal temperature on employing a controlled rate cooler
****Cyanobacteria grown on agar slope in cryotube, overlayered with cryoprotecrant

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Table 6 Reports of successful cryopreservation of Macroalgae with storage at -196ºC

Alga	procedure	Storage	Reported viability*	Reference
Gametophytic cells of *Eisenia bicyclis*	Conventional 2-step cooling + CPA	200 days	27% Male	Kono et al. 1998
Gametophytes of *Undaria pinnatifida*	Conventional 2-step cooling + CPA			Renard et al. 1992
Sporelings and apical segments of mature thalli of the marine red alga *Gracilaria tikvahiae*	Conventional 2-step cooling + CPA	>1h		van der Meer & Simpson, 1984
Gracilaria foliifera			Thallus 36%, Sporelings 43%	
Devaleraea ramentaceae			Thallus 100%, Sporelings 100%	
Palmaria palmata			Thallus 100%, Sporelings 100%	
Chondrus crispus			Thallus 70% Sporelings 86%	
Ulva lactuca			Thallus 100%	
Enteromorpha intestinalis thallus	Conventional 2-step cooling + CPA	>1 week	100%	Fleck, 1998
Gametophytic thalli of *Porphyra yezoensis*.	One step plunge in cryostraw + CPA		>80%	Choi et al. 2013
Protoplasts of *Porphyra yezoensis*	Conventional vitrification with a range of vitrification solutions	2 days	67%	Liu et al. 2004
Undaria pinnatifida gametophytes	Encapsulation dehydration with PVS2, vitrification		31% Male	Wang et al. 2011
Gametophytes of *Laminaria digitata*	encapsulation dehydration in sucrose followed by slow cooling & plunge		25-75%	Vigneron et al. 1997

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Table 6 cont.

***In many cases these data are on the basis of positive assessment using a vital and/or a mortal stain to differentiate between live and dead cells rather than the capacity of individual spores, cells or thalli to regrow. Where available data are from samples assessed at least 24 hours after thawing/rewarming, as materials immediately post-thaw will inevitably provide an over-estimate of viability levels. (Day, 2018)**

Alga	Procedure	Storage duration	Reported viability*	Reference
Undaria pinnatifida (gametophytes)	Conventional 2-step cooling + CPA	9 days	Survival	Arbault et al. 1990
Porphyra yezoensis	Conventional 2-step cooling + CPA	Up to 300 days	60%	Kuwano et al. 1993
Porphyra linearis	Conventional 2-step cooling + CPA	20 min at -196ºC	up to 70%	Arbault & Delanoue, 1994
Porphyra miniata	Conventional 2-step cooling + CPA	Long-term (>10 years)	Viable	Day, 1998
Vegetative thalli (apical tips) of: *Gracilaria corticata*	Conventional 2-step cooling + CPA		Viable, regeneration of thalli	Lalrinsanga et al. 2009
Ulva lobata				
Hypnea musiformis				
gametophytic thalli of *Ulva prolifera*	Conventional 2-step cooling + CPA	120 days	>90%	Lee & Nam, 2016
Laminaria japonica spores	Conventional 2-step cooling + CPA	24h	50%	Zhang et al. 2007
Ectocarpus thalli & spores	Conventional 2-step cooling + CPA	1 month	5-50%	Heesch et al. 2012
Female gametophytic cells of: *Laminaria japonica*	Conventional 2-step cooling + wide range of CPA	>12h		Kuwano et al. 2004
L. longissima			37-60%	
Kjellmaniella crassifolia			8-67%	
Ecklonia stolonifera			28-60%	
E. kurome			1-42%	
Undaria pinnatifida			1-60%	

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5. Conclusion

The scarcity of effective cryopreservation methodologies for key cell types such as germ cells and embryos is limiting the development and exploitation of this sector of science, a clear need exists to have reliable methodologies as well as strong research activity in marine cryobiology. The advances made in this JRA will form a major breakthrough that will generate immense benefits. Building on the partner's experience in cryopreservation, culture collections/ Biological Resource Centre management, biobanking of marine organisms, cell and fixed specimen preservation, and through the utilization of the findings of previous EU funded projects we can increase the knowledge in marine cryobiology exponentially and develop cryopreservation protocols for many previously under-exploited cell types.

6. APPENDICES

6.1 Appendix 1

The JRA2 Discussion Forum has been established and it is already working, below there are some screen captures and data that we have started to collect from all the partners:

👋 Questions about methodology

It would be extremely useful to know the different types of methodologies available in the different Marine Stations/Institutions. Therefore in order to gather the information I would encourage you to answer the following two practical questions about methods, you can also specify which method you use with which cell type/species or why did you select the equipment and all the information each one of you consider useful.

What kind of cryopreservation techniques/equipment do you use/have/have access to in your MBS?

1.- Slow cooling with Controlled Rate freezer
2.- Slow cooling with passive freezer
3.-Liquid nitrogen vapor freezing
4.- Vitrification
5.- Encapsulation/vitrification
6.- Vitrification & ultra-rapid warming
7.- Freeze-dry

What kind of cryopreservation containers do you normally use?

1.- Vials (2-4 ml)
2.- Eppendorf
3.- Straws (0.25-0.5 ml)
4.- Other (in this case specify which container and volume)

no	*yes*	Methodology						
INSTITUTTION	RESPONSIBLE	*Slow Freezer*	*Pasive Freezer*	*Ln vapour*	*vitrification*	*Encapsulation&vitrification*	*Vitrification&fast warming*	*Freeze-dry*
CCMAR	E. Cabrita							
Uvigo	E. Paredes							
UPV/EHU	I. Cancio							
UPMC	I. Probert							
NIOZ	H. Bulhuis							
SAMS	J.Day							
MBA	A. Ward							
USTAN	I. Samorjai							

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no	*yes*	Volumes/containers			
INSTITUTTION	RESPONSIBLE	*2-4 mlVial*	*Eppendorf*	*Straws 0.25-0.5 ml*	*Other*
CCMAR	E. Cabrita				
Uvigo	E. Paredes				cryotops/cryoloops
UPV/EHU	I. Cancio				
UPMC	I. Probert				
NIOZ	H. Bulhuis				
SAMS	J.Day				
MBA	A. Ward				
USTAN	I. Samorjai				

Topic: storage capabilities

[redacted]

The answers for us here in Roscoff:

For storage of cryopreserved samples we use both liquid N2 and a -150°C freezer.

Hi ther[redacted]

we use the following cryopreservation techniques/equipment

For storage of cryo-preserved samples we use a -150°C freezer.

[redacted]

I omitted to say we have for the past 40 years used liquidphase LN2 storage. In the past we had vessels that rapidly boiled off so much of the storage time the vials were in vapour phase, but the inventory was always (nearly always) in direct contact with liquid nitrogen. Under normal conditions temperatures would not have exceeded -190oC.

Topic: selected cryo containers

[redacted]

1.- Vials (2-4 ml)

2.- Eppendorf

3.- Straws (0.25-0.5 ml) Never used but we want to use them for fish and mollsuc sperm

[redacted]

Question 2

1.- Vials (2-4 ml)

Dec 04, 2017 at 1:05 PM 1 person applauded Notified 7 people

[redacted]

1 (Vials 2ml)

[redacted]

What kind of cryopreservation containers do you normally use?

1.- CryoVials (1-2 ml)

[redacted]

We use vials as standard.

2.- EppendorfÂ

3.- Straws (0.25-0.5 ml)

I have used these in experiments but found them more problematic to use than vials. Also, need to empiricaly revailidate protocols as cooling and ice formation will be different than for vials.

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6.2 Appendix 2

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