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CRYOMAR PROTOCOL TOOLBOX

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GENERAL DATA

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Report	☒
Websites, patent filling, etc.	☐
Ethics	☐
Open Research Data Pilot (ORDP)	☐
Demonstrator	☐
Other	☐

Dissemination Level

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Abstract

The aim of this report is to publish cryopreservation protocols developed and or standardized due to the Work Package 8 (JRA 2 CRYOMAR) coordinated research effort in cryobiology applied to the marine environment, as well as, stablishing the importance of the implementation of this biotechnology in the field of marine science alongside standardization of related protocols that have determinant influence of the quality of the cells pre-cryopreservation.

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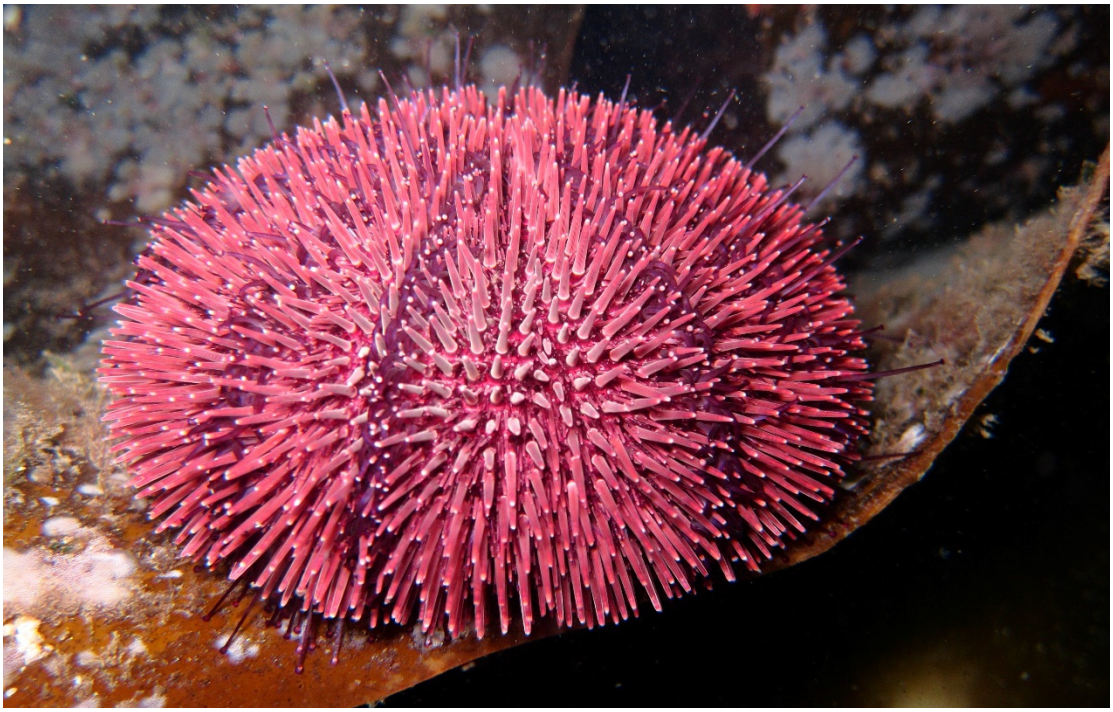
CRYOPRESERVATION OF MARINE INVERTEBRATES



Sphaerechinus granularis at Ria de Vigo (Spain) by Alicia Sobrino.

The cryopreservation of marine invertebrate organisms has been reduced to a limited number of species and cell types, in contrast to the cryopreservation research of mammalians, fish or plants. Oysters and sea urchins were the initial targets of the first studies in the 70`s but then marine organisms fell into a relative oblivion until the 90`s. While cryopreservation has been used for many decades in land animal husbandry, cryopreservation of marine organisms is developing slowly and regarding marine invertebrates barely over 50 different species have cryopreservation protocols developed (e.g. Adams et al. 2011, Suquet et al. 2014). Oysters are the best studied due to their global economic importance. Recent work published indicates that marine invertebrate cryopreservation is significantly progressing. In the past, there had been a general lack of standardization among studies, failing to report certain parts of the procedure that ended up being key to replicate the experiments or which is the quality control of the cells prior and after cryopreservation (Paredes 2015).

The following protocols aim to propose standard protocols for obtaining the gametes, quality assurance of the cells prior cryopreservation, cryopreservation protocols explained step by step and the evaluation of cell viability post thaw. The fields of application of cryopreservation of marine organisms have extensively increased along the years, from the use in breeding industry, conservation of endangered species, DNA or germplasm biobanking and marine research in general as it is the only method for long-term conservation of cells that ensures genetic stability along time (Zhang 2014).

Biobanking of marine biological resources is not a new idea but it had mostly been considered regarding microorganisms (algae, fungus, bacteria) and fish (mostly fish sperm) but up until the last 10 years the idea of creating a biobank of marine invertebrates was something quite new. The Hagedorn group at the Smithsonian Institution (USA) has been a pioneer in this matter and had established genetic banks for corals, successfully

freezing coral sperm and larvae. These biobanks have demonstrated that cryopreserved cells can be used for selected breeding, hybridization, and other applications in the conservation of coral reefs. Cryopreservation and biobanking are the next biotechnology that will change how marine science is done, as having access to biological material in a constant and reliable way is the base of scientific development.

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References:

Adams, S.L., Smith, J.F., Tervit, H.R., McGowan, L.T., Roberts, R.D., Achim, R.J., King, M.G., Gale, S.L. Webb, S.C. Cryopreservation of molluscan sperm: oyster (Crassostrea gigas, Thunberg), mussel (Perna canaliculus) and abalone (Haliotis iris). In: Cryopreservation in Aquatic Species, 2nd Edition. T. R. Tiersch and C. C. Green (Eds.) World Aquaculture Society (2011) Baton Rouge, Louisiana, pp: 562-573.

Paredes, E. Exploring the evolution of marine invertebrate cryopreservation - landmarks, state of the art and future lines of research. Cryobiology 71 (2015), 198-209. 10.1016/j.cryobiol.2015.08.011

Suquet, M., Labbé, C., Puyo, S., Mingant, C., Quittet, B., Boulais, M., Queau, I., Ratiskol, D., Diss, B., Haffray, P. Survival, growth and reproduction of cryopreserved larvae from a marine invertebrate, the Pacific oyster (Crassostrea gigas). PLoS ONE 9, 4 (2014), 0093486. doi.org/10.1371/journal.pone.0093486

Zhang, T. Cryopreservation of gametes and embryos of aquatic species. In: Life in the frozen state. B.J. Fuller, N. Lane, E.E. Benson (Eds.) CRC Press (2004), 415-136.

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MUSSEL SPAWNING BY THERMAL CYCLING

1. Prepare a plastic box (preferably not white) with filtered Sea water and heaters to achieve 20ºC.
2. Place 50 to 100 mussels (previously roughly cleaned from barnacles or algae growths on their shells), dry into a tray at 4ºC for 10-15 minutes.
3. Transfer those mussels to the box conditioned to 20ºC and spread the mussels so you can clearly see each individual.
4. Monitor for spawning.

Figure 1.- Mussel (*Mytilus galloprovincialis*) spawning

5. When a spawning mussel is located, quickly retire the mussel to an individual beaker with clean filtered sea water in the case of females.
6. Males can be placed into a beaker with water or sperm can be collected dry and concentrated by placing the mussel into a dry beaker.

Figure 2.- Female mussel on the left, male mussel on the right.

MATERIALS & REAGENTS

50-200 Mussels

Plastic Box

Heaters

Plastic tray

Thermometer

Portable light

Individual beakers

Filtered Sea Water

Microscope

QUALITY CHECK OF THE GAMETES:

Oocytes should present spherical and homogeneus shape and brown-ish dark colour.

Sperm: >80 % motile

Fertilization: oocyte-sperm ratio is 1:10 allowing a 20 minute contact time.

7. If once placing the mussels into the box no spawning takes place in 30 minutes, repeat the exposure to 4ºC (step 2) and return them to the 20ºC spawning box. Repeat as necessary.
8. In combination with Thermal cycling, food presence has been reported as stimulating too, microalgae can be added into low concentration to the box.

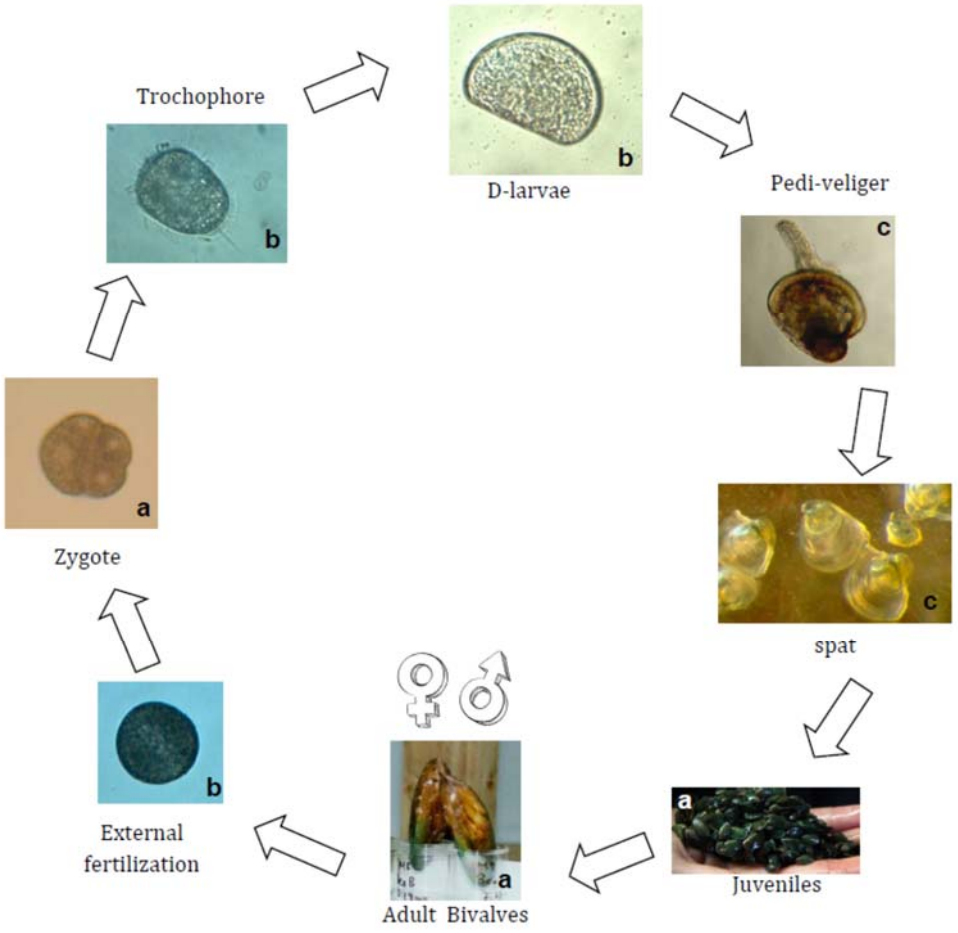

Figure 3.- General development of mussels. Trocophores can be obtained at 18-24 hours post fertilization (18-20ºC), larvae-D appears after 48 hours. Larval rearing lasts around 22 days until larvae are ready for settlement. Pictures correspond to different types of mollusks.

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.- http://www.fao.org/fishery/culturedspecies/Mytilus_galloprovincialis/en

2.- Gosling, E. Bivalve Molluscs: Biology, Ecology and Culture. Wiley publications 2003

3.-Cultivo del mejillon, Observatorio Español de Acuicultura http://www.observatorio-acuicultura.es/sites/default/files/images/adjuntos/libros/cuaderno_mejillon.pdf

MUSSEL LARVAE CRYOPRESERVATION

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MUSSEL LARVAE CRYOPRESERVATION

1. Spawn mussels and check gamete quality: eggs round, homogeneous shape and good colour. Sperm is checked for motility.

Figure 1.- Female mussel on the left, male mussel on the right.

2. Induce fertilization by adding sperm to the oocytes in a ratio 10:1 or 20:1, allow 15 minute contact time while mixing gently from time to time.
3. Check for % of fertilization, either by counting the percentage showing polar bodies or later checking cell division. Transfer the fertilized oocytes to tanks for incubation until desired larval stage. Incubation density from 40 to 80 larvae/mL.
4. Monitor for development. Once the development stage is optimal, filter the cells using a sieve with water to concentrate them gently. Collect the larvae into a measure cylinder. Check for larval health indicators like shape and movement. Calculate density.
5. Prepare the biofrezer with the following protocol programmed: Start at 4ºC, hold for 2 minutes, and cool at 1 º C/min to -12, hold for two minutes for seeding. Cool at 1ºC/min to -35ºC, hold 2 minutes and transfer the straws to liquid nitrogen for storage.
6. Prepare a cryoprotectant solution of the double of the concentration required for cryopreservation. Add in a proportion 1:1 volumes of larvae and cryoprotectant solution so the final concentration is 10% Ethylene glycol + 0.4M trehalose. Allow an equilibration time of 15 minutes.
7. During those 15 minutes, load the larvae in the straws by aspiration. Pin them in the comb and seal them with sealing powder. Clean the straws and place in water by the sealing side to harden. Dry before placing them in the biofrezer.

MATERIALS & REAGENTS

50-200 Mussels, Plastic Box and Heaters for induction.

Microscope, measure cylinder, equipment for larval rearing.

Biofreezer , Straws, sealing powder.Combs and holder for straw filling.

Small scissors

Liquid nitrogen (LN2)

Ethylene glycol 20% (v/v) + 0.8M trehalose solution in Filtered Sea Water or artificial sea water.

QUALITY CHECK OF THE GAMETES:

Oocytes should present spherical and homogeneus shape and brown-ish dark colour.

Sperm: >80 % motile

Fertilization: oocyte-sperm ratio is 1:10 allowing a 20 minute contact time.

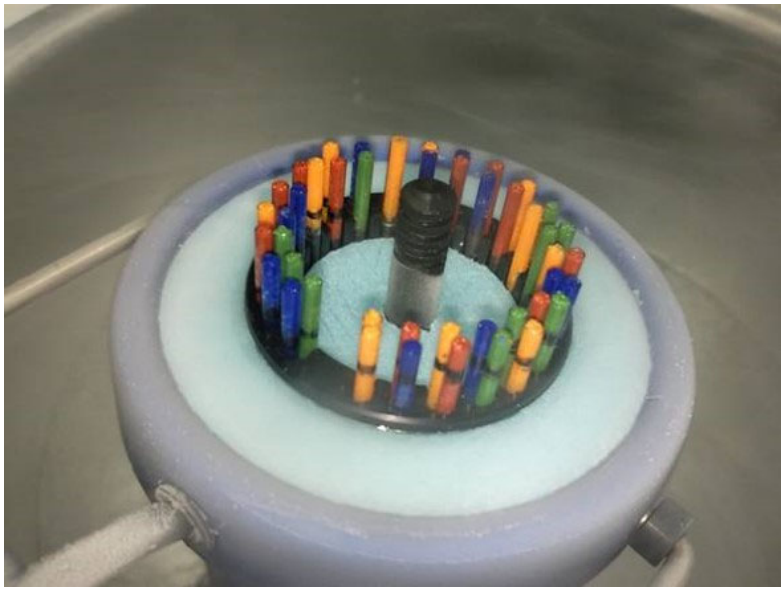

Figure 2.- Biofreezer from Cryologic Ltd. (Australia) with 0.25 mL straws

8. After 15 minutes, place the straws in the biofreezer (sealed side up) and run the protocol.
9. For thawing, transfer one by one the straws to a warm water bath of 35ºC for 6 seconds (the time the straw takes to melt the ice and become transparent).
10. Cult one side of the straw and place over a container before cutting the other side and resealing the larvae from the straw.
11. Culture those larvae until desired endpoint.

WARNINGS

[1]THIS PROTOCOLS ALLOWS 50% OF CRYOPRESERVED TROCHOPHORE LARVAE TO D-LARVAE POST-THAW (48 H).

[2]ONLY 1% OF THE CONTROLS SURVIVE THE 22 DAYS LARVAL REARING. SETTLEMENT OF THE CRYOPRESERVED LARVAE IS ALMOST 70% OF THE CONTROLS.

[3]THIS PROTOCOLS ALLOWS FOR OVER 90% D-LARVAE (72H OLD) TO SURVIVE 48 HOURS POST-THAW. THERE IS NO DATA AVAILABLE FOR LONG TIME INCUBATION.

DURING WARMING IF EXCEEDING THE 6 SECONDS, THE TEMPERATURE OF THE STRAW WILL EQUILIBRATE WITH THE 35ºC WATER BATH AND LARVAE WILL DIE.

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.-Paredes, E., Bellas, J., Adams, S.L. Comparative cryopreservation study of trochophore larvae from two species of bivalves: Pacific oyster (Crassostrea gigas) and blue mussel (Mytilus galloprovinciallis). Cryobiology 65, (2013),256-262.

2.- Rodríguez, R., Heres, P., Troncoso, J., Paredes, E. Long term development of cryopreserved mussel (Mytilus galloprovincialis) larvae. Aquaculture 512, (2019), 734326

3.- Heres, P., Rodriguez-Ribeiro, R., Troncoso, J., Paredes, E. Toxicity tests of cryoprotecting agents for Mytilus galloprovincialis (lamark, 1819) early developmental stages. Cryobiology 86, (2019), 40-46.

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MATERIALS & REAGENTS

Styrofoam box with lid

Floating rack of 5 cm

0.25 mL straws

Sealing powder for straws

REACCTIVES:

DMSO solution 2.8M in FSW (18-20ºC)

Liquid nitrogen (LN2)

QUALITY CHECK OF THE GAMETES:

Oocytes should present spherical and homogenous shape and brown-ish dark colour.

Sperm: 100% motile before cryopreservation and over 60% motile post-thaw (although slower)

Fertilization:

Normal sperm oocyte rate is 20:1 for 10-20 minute contact time at 20ºC. With cryopreserved sperm 100:1 sperm ratio yields around 50% fertilization success.

MUSSEL SPERM CRYOPRESERVATION:

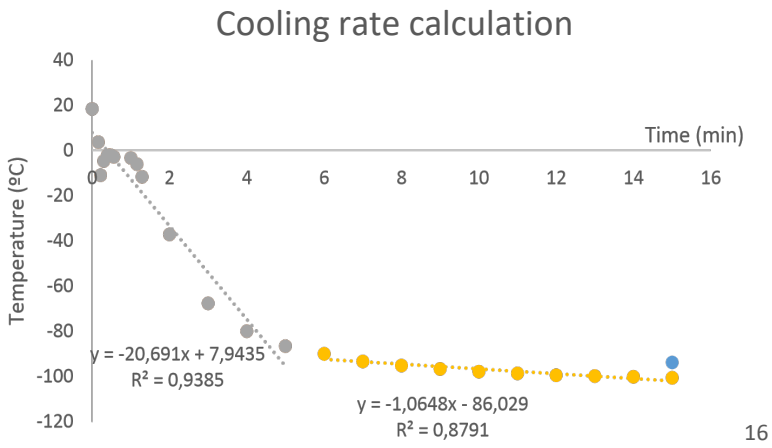
1. Spawn mussels according to Mussel spawning protocol
2. When a spawning mussel is located, quickly retire the mussel to an individual beaker dry in case of males and with clean filtered sea water in the case of females
3. Males can be placed into an empty beaker so sperm can be collected dry and concentrated.

Figure 1.- Styrofoam box and floating rack example

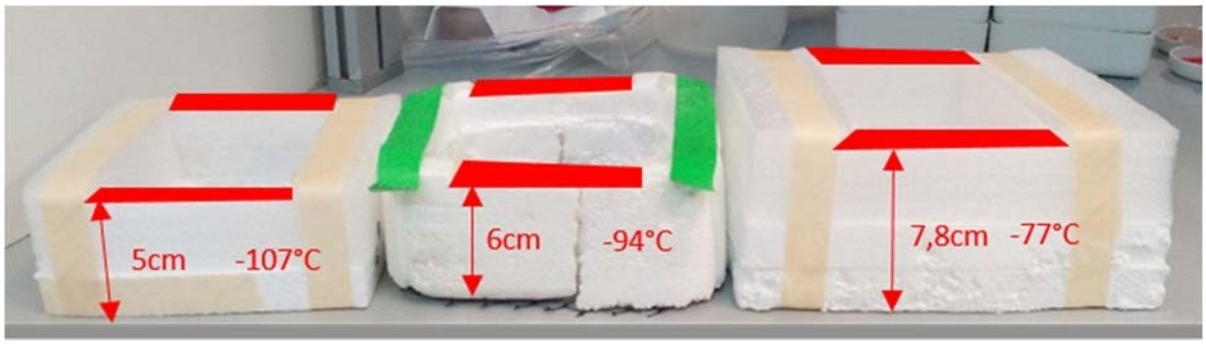
4. Dilute the pure sperm with the DMSO solution 1:1 and aspire the mixture into 0.25 mL straws. Final concentration 1.4M (10% v/v).
5. Allow equilibration time of 5 min room temp (18-20ºC) 6. Place the straws into the floating rack in a polystyrene box 5 cm above the liquid nitrogen level for 8 min.
7. Plunge into liquid nitrogen. Store in Liquid nitrogen as needed.
8. Thaw the straws into a water bath 35ºC (6 seconds)
9. Dilute the CPA with FSW slowly to avoid osmotic shock (4% FSW addition in each step) until motility is regained.
10. Assess motility and fertilization.

MUSSEL SPERM CRYOPRESERVATION PROTOCOL

How was cooling rate calculated:



Although traditionally cooling in liquid nitrogen vapour was done for 15 minutes. We calculated the cooling rate and determined that the fast cooling only happened in the first 6 minutes, then the cooling was lower than 1ºC/min. Using 8 minutes and the plunging point to liquid nitrogen (LN2) resulted in better sperm movement. Cooling rate during those 8 minutes is approximately 20ºC/ min. (Ending temperature is around 100ºC when plunged to LN2 for conservation).



Home-made racks examples. In red you can see the rack height and the respective final temperature the samples can reach.

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.- Liu, B., Liu, Y., Liu, S., Xu, T., Liu, Q.,Li, X. Cryopreservation of strip spawned sperm using non-programmable freezing technique in the blue mussel Mytilus galloprovincialis. Aquaculture Research 47, (2015),3888-3898

2.- Di Mateo, O., Langellotti, A.L., Masullo, P., Sansone, G. Cryopreservation of the Mediterranean mussel (Mytilus galloprovincialis) spermatozoa. Cryobiology 58, (2009),145-150.

PORTUGUESE OYSTER (*Crassostrea angulata*) SPERM CRYOPRESERVATION PROTOCOL

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MATERIALS & REAGENTS

Programable biofreezers (6 ºC/min)

French straws 0.5 ml

Freezing solution: filtered artificial seawater with 20% DMSO

Liquid nitrogen

QUALITY CHECK OF THE GAMETES:

Sperm motility and plasma membrane viability

Sperm: >40 % motile

SPERM COLLECTION AND STORAGE OF PORTUGUESE OYSTER

1- Open oysters using a knife and collect a sample from the gonad to determine sex microscopically.
2- Collect sperm by dry method, performing small incisions in the gonad and collect the sperm sample with a micropipette into an eppendorf tube. Add 100 µl of artificial sea water to the tube and resuspend the cells.

3- Wash and filter sperm immediately with a 20 and 100 µm sieves. Dilute the sample in 500 µl of artificial sea water.
4- Assess the total motility and concentration of fresh sperm. Only samples with motility higher than 40% are selected. Dilute sperm to have a final concentration between 1 to 2 x10^9 spermatozoa/ml in artificial seawater.

SPERM CRYOPRESERVATION PROCEDURE

SPERM CRYOPRESERVATION:

1- Add sperm into the freezing solution 1:1 (v:v) and load the sample into 0.5 ml French straws with 10 min of equilibration.
2- Perform a cooling rate of 6°C/min from 0 to -70°C with a programable biofreezer (Asymptote Grant EF600, UK). After freezing, the straws are directly plunged into liquid nitrogen and stored in the cryobank.
3- Thaw the straws in a water bath set at 37°C for 10 s.
4- Perform sperm quality analysis immediately after thawing, since the viability of spermatozoa decreases with post-thaw time.

SPERM QUALITY ANALYSIS

SPERM MOTILITY ASSESSMENT

1- Use CASA system settings set for 25 frames per second to evaluate sperm motility. 2- Add 1 µl of sperm to the Makler chamber.
3- Dilute sperm with 9 µl of artificial seawater in the Makler chamber.
4- Record motility parameters immediately after sperm dilution.

SPERM VIABILITY ASSESSMENT

Two methods to assess oyster sperm viability (fluorescence microscope or flow cytometer) can be used.

Fluorescence microscope

1- Mix 15 µl of diluted sperm, 0.5 µl SYBR Green (final concentration 100 nM) and 1.5 µl propidium iodide and observe in a fluorescence microscope.
2- Count at least 100 cells, distinguishing live (SYBR green positive, green cell) and dead cells (PI stained, red cells).

Flow Cytometer

1- Dilute 5 µl of sperm in 500 µl of 1% NaCl buffer.
2- Add 2 µl propidium iodide (PI) at a concentration of 2.4 mM to the suspension.
3- Analyse in a flow cytometer after 5 min incubation in the dark.

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MATERIALS & REAGENTS

SEA URCHIN SPAWNING METHODS

Figure 1.- Regular Sea Urchin *Echinus esculentus*

1. Dissection and direct collection from the gonads: Dissect the sea urchins alongside their transversal axis, then cutting all the gonads in half. If the sea urchin is mature from the dissected gonads eggs or sperm should be easy to collect with a Pasteur pipette (Figure 2).
   Sperm activates in contact with sea water, it can be collected pure and stored in the fridge for several hours. Oocytes are sensitive to drops in temperature, so storage should be done at room temperature.

Figure 2.- Dissected sea urchin pair, with reddish/orange gonads a *P. lividus* female and below with pale gonads a male.

QUALITY CHECK OF THE GAMETES:

Oocytes should present spherical and homogenous shape and brown-ish colour.

Sperm: >80 % motile

Fertilization: oocyte-sperm ratio is 1:10 . Fertilization takes a few seconds and a fertilization membrane develops allowing for a quick fertilization percentage check (Figure 3).

Non lethal sex differentiation in sea urchins: Paredes, E., Costas, D. Non-lethal sex identification of sea urchins: method and advantages. Lab Animal 49 (2020), 7-8

2. KCl injection: 1 ml of a 0.5M KCl solution can be injected into several sites through the soft membrane around the mouth (peristomial membrane). Within minutes, the gametes should appear and can be collected in water. It is not lethal per se, but post-injection mortality is high.

3. Electric stimulation: Electrical stimulation with a current of 10 V that can be passed through the shell and triggers a small spawning allows sex differentiation and gamete collection. According to the literature, the method is associated with a good survival rate post-spawning.

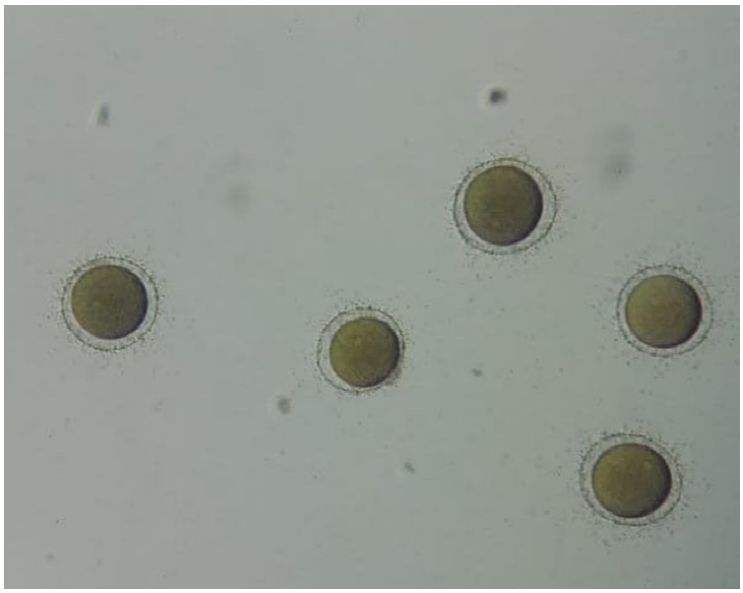

Figure 3.- *P. lividus* eggs with fertilization membranes and an excessive amount of sperm per egg. The fertilization membrane has the role of protecting against polyspermy but when a big amount of sperm is added it can still lead to polyspermy despite this natural protection.

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.- Conway, C.M., Igelsrud, D. and Conway, A.F. In C. L. Harris (Ed.). Proceedings of the third workshop/conference of the Association for Biology Laboratory Education (ABLE). Kendall/Hunt Publishing Company, Dubuque, Iowa. (1984).

2.- Lawrence, J.M. Sea Urchins, Volume 38: Biology and Ecology (Elsevier: Amsterdam, The Netherlands 2007).

3.- Foltz, K.R., Hamdoun, A. Echinoderms Pasrt A- Methods in Cell biology 2019 , Volume 150.

4.- Paredes, E., Costas, D. Non-lethal sex identification of sea urchins: method and advantages. Lab Animal 49 (2020), 7-8 10.1038/s41684-019-0439-y

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SEA URCHIN EMBRYO CRYOPRESERVATION:

1. Obtain sperm and eggs from *Paracentrotus lividus* using a spawning method form the spawning protocol. Fertilize the eggs with a 10:1 sperm/egg ratio and incubate for 8 hours (18ºC) until early blastula stage.
2. Filter and concentrate the blastulas using a 40 µm mesh
3. Place 1 mL of blastulas in a 2 mL cryovials and add the cryoprotectant solution (CPAs) following the proportions cited in the table below in equimolar steps 1 minute apart (at room temperature, 18ºC). CPAs are always prepared doubled the final concentration required, which is Dimethyl sulfoxide (DMSO) 1.5M plus 0.04 M Trehalose (TRE) in Filtered Sea Water (FSW).

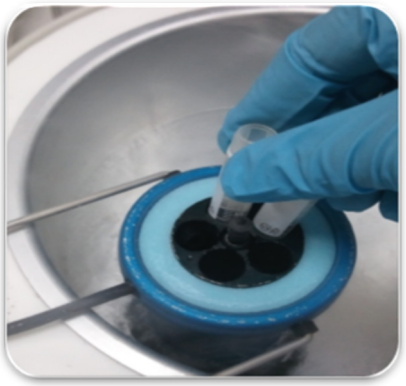

Figure 1.- Portable Controlled-rate freezer Cryologic Ltd.

4. Once the cryoprotectant has been loaded in the vials, place the vials into the freezer and run the embryo preservation protocol (Fig. 1).
5. Programmable controlled-rate freezer: ºC for 2 min, and then cooled at a rate of 1 ºC min-1 to -12 ºC. At this point vials were seeded during a 2 min hold, followed by cooling at 1 ºC min-1 to – 80 ºC. A final hold of 2 min was placed at 80 ºC and vials can be quickly transferred to liquid nitrogen for storage.
6. Thawing is performed by immersion into a 17 ± 1 ºC water bath until the ice is completely melted.
7. CPAs are now removed with clean FSW in 12 equimolar steps one minute apart (See table below) at room temperature 19 ± 1 ºC and embryos were finally rinsed with clean FSW.

MATERIALS & REAGENTS

2 mL cryovials

40 µm filters

REACCTIVES:

Cryoprotectant: 3M DMSO + 0.08M Trehalose in Sea Water

Liquid nitrogen (LN2)

QUALITY CHECK OF THE GAMETES:

Oocytes should present spherical and homogeneus shape and brown-ish dark colour.

Sperm: >80 % motile

Fertilization: oocyte-sperm ratio is 1:10. Fertilizatio occurs within seconds and only use batches with fertilization success over 90%

Blastulas post-thaw should maintain their shape an integrity. Development will slow down in the first hours post-thaw and 4-arm pluteus larvae should appear by 96 hours.

Steps of 1 minute	Cryoprotecting solution addition procedure (µL of CPA)	Dilution procedure (µL of FSW)
1	35	143
2	37	165
3	40	193
4	43	228
5	46	273
6	50	334
7	55	417
8	60	536
9	65	715
10	72	1000
11	79	1500
12	88	2500
13	98	
14	111	
15	125	
Total time (min)	15 minutes	12 minutes

BIBLIOGRAPHY FOR FURTHER CONSULT:

1. *Paredes, E., Bellas, J., D. Costas. Sea urchin (Paracentrotus lividus) larval rearing - culture from cryopreserved embryos. Aquaculture 437 (2015), 366-369.* doi.org/10.1016/j.aquaculture.2014.12.022
2. *Paredes, E., Bellas, J. The use of cryopreserved sea urchin embryos (Paracentrotus lividus) in marine quality assessment. Chemosphere 128 (2015), 278-283* 10.1016/j.chemosphere.2015.02.007
3. *Paredes, E. Biobanking of a marine invertebrate model organism: The sea urchin. Journal of Marine Science and Engineering (2016)* doi.org/10.3390/jmse4010007
4. *Paredes, E., Bellas, J. The use of cryopreserved biological material for water quality assessment. Frontiers in Marine Science (2019)* doi.org/10.3389/fmars.2019.00454

E. Paredes

Centro de Investigación Mariña – ECIMAT Marine Station - Universidade de Vigo

MATERIALS & REAGENTS

SEA URCHIN SPERM CRYOPRESERVATION:

1. Obtain sperm using one of the methods listed on the spawning protocol.

Figure 1.- Dissected sea urchin pair, with reddish/orange gonads a *P. lividus* female and below with pale gonads a male.

2. Sea urchin sperm activates when in contact with sea water. Collect the sperm as concentrated as possible until cryopreservation. Keep in the fridge for storage longer than 30 minutes.

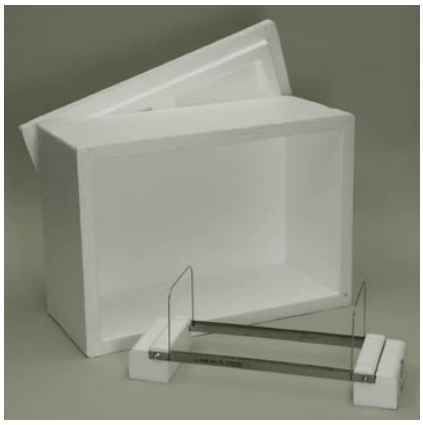

Figure 2.- Styrofoam box and floating rack example

3. Dilute the pure sperm with the DMSO solution 1:1 and aspire the mixture into 0.25 mL straws. Final concentration 15% (v/v).

Styrofoam box with lid

Floating rack of 5 cm

0.25 mL straws

Sealing powder for straws

REACCTIVES:

DMSO solution30% (v/v) in FSW (18-20ºC)

Liquid nitrogen

QUALITY CHECK OF THE GAMETES:

Oocytes should present spherical and homogenous shape and brown-ish dark colour.

Sperm: 100% motile before cryopreservation and over 60% motile post-thaw (although slower)

Fertilization:

Normal sperm oocyte rate is 10:1 and fertilization takes a few seconds. Feritlization can be easily assessed visually with the formation of the fertilization membrane.

4. Allow equilibration time of 5 min room temp (18-20ºC)
5. Place the straws into the floating rack in a polystyrene box 5 cm above the liquid nitrogen level for 8 min (Fig 2).
6. Plunge into liquid nitrogen. Store in Liquid nitrogen long as needed.
7. Thaw the straws into a water bath 35ºC (6 seconds)
8. Dilute the CPA with FSW slowly to avoid osmotic shock (4% FSW addition in each step) until motility is regained.
9. Assess motility and fertilization.

The results are variable among sea urchin species, in all cases DMSO was the most suitable CPA but the distance of the straws from the liquid nitrogen (a.k.a cooling rate, see mussel sperm cryopreservation protocol) can vary and should be optimized for each species. This protocol was tested with P. Lividus, E. cordatum, E. esculentus, S. granularis with variable results (see fig.3).

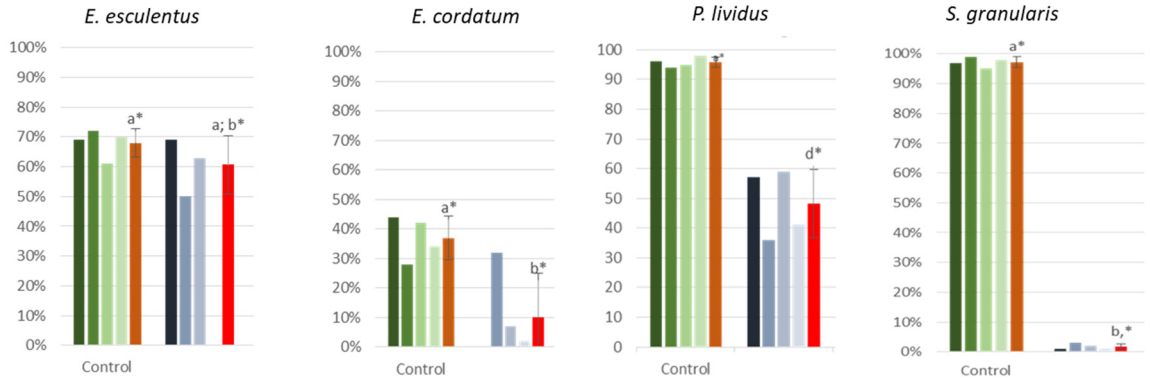

Figure 3.- Percentage of motility of fresh controls and post-thaw samples for three regular and one irregular sea urchin, following the protocols specified above and 1-1.5M DMSO.

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.-Adams, S.L., Hessian, P.A.V. Cryopreservation of Sea Urchin (Evechinus Chloroticus) Sperm. Cryoletters, Volume 25, 4 (2004), 287-299.

2.- Fabbrocini, A., D'Adamo, R., Pelosi, S., Oliveira, L.F.J., Silvestri, F., Sansone, G. Gamete cryobanks for laboratory research: Developing a rapid and easy-to-perform protocol for the cryopreservation of the sea urchin Paracentrotus lividus (Lmk, 1816) spermatozoa. Cryobiology 69, Issue 1, (2014), 149-156. doi.org/10.1016/j.cryobiol.2014.06.009

3.- Paredes E., Adams SL., Vignier J. Cryopreservation of sea urchin sperm and early life stages. Methods Cell Biol. 150 (2019), 47-69. 10.1016/bs.mcb.2018.11.008

SEA CUCUMBER GAMETE COLLECTION AND CULTURE

E. Paredes

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MATERIALS & REAGENTS

1. Dissect the sea cucumbers (*Holothuria forskali*), locate a male (gonad has white color- pale yellow, Fig 1).

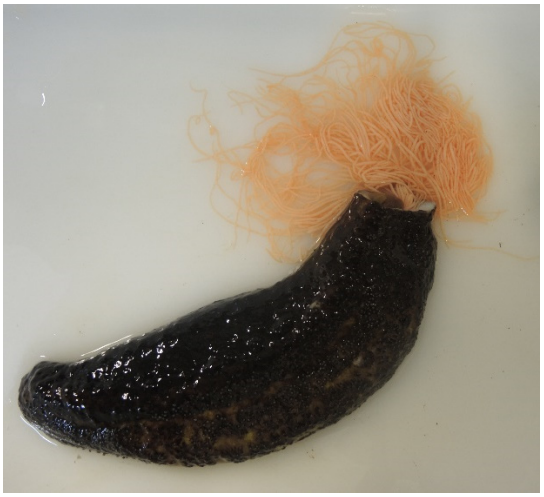

Figure 1.- Detail of the male gonad. Picture provided by T. Ballesteros and A. Tubio (ECIMAT).

2. Break the gonad and recover sperm as pure and concentrated as possible in a test tube.
3. Dilute a sample of pure sperm with 15% DMSO in filtered sea water (FSW).
4. Allow 5 minute equilibration (room temperature, 18ºC) time while loading the samples into 0.25 mL straws. After the equilibration time place on a floating Styrofoam rack (6 cm over liquid nitrogen vapor) for 30 minutes and store in liquid nitrogen.
5. Thawing is done in a water bath 28ºC for 6 seconds.
6. Dilute 1:1 the cryopreserved sperm with sea water and check motility.

QUALITY CHECK OF THE GAMETES:

The motility post-thaw is very similar to the controls.

MATERIALS:

Styrofoam box with lid

Floating rack of 5 cm

0.25 mL straws

Sealing powder for straws

REACCTIVES:

DMSO solution 15% (v/v) in FSW

(18-20ºC)

Liquid nitrogen

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.- Ballesteros, T., Tubío, A., Hernández, A., Rodríguez, R., Martínez, A., Gómez, R., Costas, D., Poza, E. & Troncoso, J. (September 2019). Reproductive cycle of the sea cucumber Holothuria forskali in the Ría de Vigo: application to fisheries management. In P. Gomes (Presidency), XX Simposio Ibérico de Estudos de Biología Marinha (SIEBM). Braga, Portugal.

2.- Laguerre, H., Raymond, G., Plan, P., Améziane, N., Bailly, X. & Le Chevalier, P. (2020). First description of embryonic and larval development, juvenile growth of the black sea-cucumber Holothuria forskali (Echinodermata: Holothuroidea), a new species for aquaculture in the North-Eastern Atlantic. Aquaqculture (online).

ALGAL CULTURE COLLECTIONS - BIOBANKS FOR SEAWEED AQUACULTURE

Green seaweeds at CCAP by SAMS

Seaweed farming is one of the fastest growing sectors of global aquaculture, producing around 30 million tonnes per year worth billions of US dollars. For seaweed aquaculture to be a successful industry, however, it needs to develop sustainable production management strategies. A key step in this process is the stable and long-term storage of living cells and the capacity to conserve genetic diversity for breeding programs as seedstock for onward cultivation.

At the Culture Collection of Algae and Protozoa (CCAP) a method has been established for cryopreserving gametophytes of *Saccharina latissima* (Visch et al 2019). This technology is being implemented as part of the EU Horizon 2020 GENIALG project, with CCAP working on establishing a seaweed "seedbank" to ensure that the wild genetic diversity of the kelp *Saccharina latissima* is documented, conserved, and made publically available for biotechnological and research purposes alike. This pan-European seedbank will ensure not only the maintenance of a diverse number of strains, but also the preservation of their functionality and, when technically possible, genetic stability. *S. latissima* samples were collected from defined geographic zones and clonal gametophytes isolated to be progressively accessed in the Collection, together with efforts to develop cryopreservation protocols, guarantee strain stability and increase the robustness of maintenance protocols. Phenotyping and genotyping data will be gathered and made available to users worldwide via the CCAP Knowledgebase.

Cryopreservation methods have also been developed for other species which are cultivated, such as the red algae *Porphyra* (nori) and the green sea lettuce *Ulva lactuca*. Algal culture collections can offer safe and bio-secure storage for these commercially valuable strains. Other brown algae such as *Ectocarpus* (Heesch et *al* 2012) and a range of Arctic brown seaweeds in the CCAP holdings have been cryopreserved using similar methods.

With intensive farming, often comes disease and this has occurred especially with seaweed farming in tropical areas. The GlobalseaweedStar project at the Scottish Association for Marine Science (SAMS) has been isolating

and characterising novel algal pathogens of macroalgae and these are also maintained in the Collection to support research into the mechanisms of pathogenesis. Such diseases can have severe economic impacts in established farms. The Globalseaweed website has further information on macroalgal diseases.

Christine Campbell & Cecilia Rad-Menéndez

Scottish Association for Marine Science (SAMS)

References

Visch, W., Rad-Menéndez, C., Nylund, G.M., Pavia, H., Ryan, M.J., Day, J. Biopreservation and Biobanking 17 (2019), 378–386. https://doi.org/10.1089/bio.2018.0147

Heesch, S., Day, J.D., Yamagishi, T., Kawai, H., Müller, D.G., Küpper, F.C. Cryopreservation of the model alga Ectocarpus (phaeophyceae). CryoLetters 33 (2012),327–336

https://genialgproject.eu/

https://www.globalseaweed.org/

CULTIVATION OF MACROALGAE IN CULTURE COLLECTIONS

C.N. Campbell, J. Field

CCAP, Scottish Association for Marine Science, Oban, UK

BACKGROUND

The Culture Collection of Algae and Protozoa (CCAP) holds around 500 strains of red, green and brown seaweeds or macro-algae. The easiest method of maintenance is cryopreservation but, as yet, this is not possible for all strains. For most species it is optimal to grow, slowly, small amounts of biomass at low temperature and low light.

In CCAP we culture unialgal strains, most of which also have bacteria present. As the cultures are relatively clean there is no need to add germanium dioxide to inhibit diatom growth as is often the practice in seaweed farm 'hatcheries'. For maintenance purposes the seaweeds are grown vegetatively. For some, such as the kelp *Laminaria*, it is preferable to culture small gametophytes.

1. **Vessel:** Depending on the size of the thallus (clumps) they are grown in either test tubes, small Petri dishes or tissue culture flasks, small glass flasks or large (1 litre) glass flasks.
2. **Temperature:** Polar strains are grown at 3°C, temperate strains at 12°C and tropical strains at 20°C.
3. **Medium:** We use either Modified Provasoli or NSS (low).
4. **Lighting:** Low light from either a fluorescent tube or LED supply and for *Saccharina latissima*, for instance, red light is used. Plastic film can be used to shade the light.
5. **Culture Period:** Some strains can be survive well for up to six months or longer without transfer to fresh medium. This is especially true of the polar or temperate strains.
6. **Culture checking:** before beginning to transfer, examine your culture carefully, preferably using a stereo microscope. You should select the most healthy, well-pigmented clumps with good morphology.
7. **Aseptic technique** must be used throughout, working in a clean laminar flow cabinet with sterile equipment and media.
8. **Culture Method:** If the culture is made up of a large number of small clumps, then select a couple and transfer them to fresh medium in a new vessel. This can be done with a sterile pipette or with forceps. It is more likely that you will have to remove a fragment of a clump. You may have to pick out a clump of thallus and transfer it to a beaker or clean Petri dish for the next stage. Dissect with sterile forceps, teasing a fragment apart from the larger clump or cut a piece of thallus with a sterile scalpel. Transfer to the fresh medium, close the vessel with a screw top, bung or seal a Petri dish lid with Parafilm.

MATERIALS & REAGENTS

Macroalgal Cultures

Sterile medium

(CCAP Media recipes:

https://www.ccap.ac.uk/pdfrecipes.htm)

Appropriate vessel

Filtered Sea Water

Sterile beakers

Sterile forceps and scalpel

Sterile pipettes

Sealing tape (Parafilm)

Stereo Microscope

Constant temperature rooms or incubators at required temperature

Lighting: low lighting, either daylight or red.

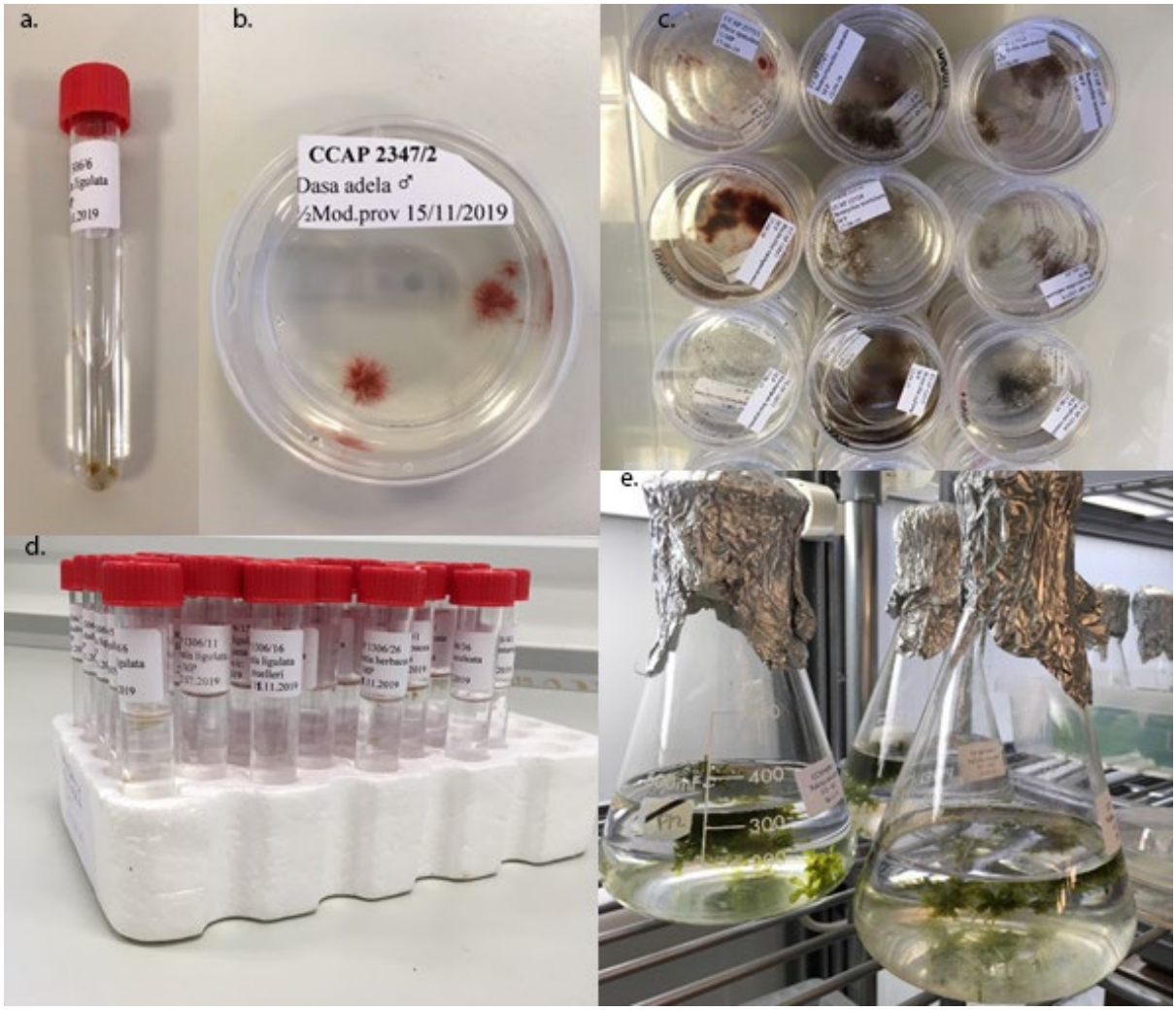

Fig 1 Examples of macrolagal cultures in CCAP: a. Small clump of *Desmarestia* in plastic tube. b. Group of clumps of the small red epiphyte, *Dasa adela*. c. Multiple small Petri dishes of small red seaweeds sealed with tape. d. Rack of multiple samples of small brown filamentous seaweeds. At low temperature and in low light, these will remain viable for over 1 year. e. Large flasks of green seaweeds.

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.- CCAP website catalogue knowledgebase: https://www.ccap.ac.uk/knowledgebase.htm

2.- Kawai, H., Motomura, T., Okuda, K. (2005) Isolation and Purification techniques for macroalgae. In Algal Culturing Techniques ed Andersen RA. Academic Press.

CRYOPRESERVATION OF A RED SEAWEED

J. Field, C. Campbell

Culture Collection of Algae and Protozoa, SAMS, Scotland, UK

CRYOPRESERVATION METHOD FOR *PORPHYRA UMBILICALIS*

(MODIFIED FROM KUWANO *ET AL* 1996)

1. *Porphyra* thalli and blades should be in good condition, unialgal and in growing phase (see photo).
2. As the alga is kept at 8 °C, store materials and chemicals at this temperature prior to use.
3. Aseptically cut the thalli into small pieces and transfer them to a sterile beaker.

Flask with thallus of *Porphyra umbilicalis*

4. Add 10 mls culture medium then 10 mls of cryoprotectant solution. Do this slowly over a 15 minutes period.
5. Leave for 45 minutes at 8 °C.
6. Put small piece of thallus into 2ml cryotube and top up with the cryoprotectant medium.
7. Place tubes in controlled rate freezer and use programme to reduce temperature by 1 °C/minute from 8 °C to -40 °C and hold the temperature at -40° for 15 minutes.
8. Plunge into liquid nitrogen in Dewar.
9. Thaw at 37 °C until all the ice has melted, immediately wash off cryoprotectant using cooled sterile seawater.
10. Place into normal growth medium and vessel, maintain in dark for 24 hours, then into moderate light.

REFERENCES:

1.-Day, J.G. Cryopreservation of Microalgae and Cyanobacteria. In: Day JG & Stacey GN (eds) Cryopreservation and Freeze-drying Protocols. Humana Press (2007), 139-149

2.-Kuwano, K., Aruga, Y., Saga, N. Cryopreservation of clonal gametophytic thalli of Porphyra. Plant Science 116 (1996), 117-124. doi.org/10.1016/0168-9452(96)04380-4

MATERIALS & REAGENTS

Cell incubators

Class I biological safety cabinet

Programmable, controlled rate cooler (Planer)

Small (1-l)Dewar

Heated water-bath

Storage cryostat/refrigerator

Long forceps

Cell culture medium: f/2 or other appropriate medium.

Solutions:

Cryoprotectant solution5% (v/v) dimethyl sulphoxide (DMSO); 5% polyvinylpyrrolidone (PVP) in sterile, filtered seawater

70% (v/v) ethanol; liquid nitrogen.

Plasticware: membrane filters (0.5µm pore size); Universal tubes (20ml); disposable pipettes; 2-ml cryogenic tubes.

Glass beakers

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

CRYOPRESERVATION OF MARINE MICROALGAE

Cryopreservation has been successfully employed in the banking and maintenance of cultures of microorganisms, from bacteria to yeasts, since the onset of cryobiology. For unicellular algae (=microalgae), cryopreservation has been increasingly widely applied in recent decades, providing an alternative to the continuous culture of strains, saving time and space and increasing the capacity of culture collections while avoiding the morphological and physiological modifications that can occur during continuous active culture (Lorenz et al. 2005, Day & Brand 2005). Microalgae are not a unified phylogenetic group, but are rather an extremely diverse group of organisms, including some prokaryoyes (cyanobacteria) and representatives in several of the main lineages of the eukaryotic tree of life. While there is no such a thing as a general cryopreservation protocol that can be successfully applied to all microalgal types with guaranteed survival, there are some more or less standardized methods presented below that can be 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, cell size and cell form, culture age and presence of gas vacuoles. 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 example most dinoflagellates,

some cyanobacteria, and most cryptophytes, with no clear patterns on how to cryopreserve specific groups having been reported.

Léna Gouhier, Priscillia Gourvil, Ian Probert

Roscoff Culture Collection

Roscoff Marine Station

References

1. Lorenz, M., Friedl, T., Day. J.G. (2005). Perpetual maintenance of actively metabolizing of microalgal cultures. In: *Algal culturing techniques* (Ed. Robert A. Anderson), Elsevier Academic Press, 145-156.

2. Day, J.G., Brand J.J. Cryopreservation Methods for Maintaining Microalgal Cultures. Cryopreservation methods for maintaining microalgal cultures. In: *Algal Culturing Techniques* (Ed. Robert A. Anderson), Elsevier Academic Press (2005),165-187.

L. Gouhier, P. Gourvil, I. Probert

Roscoff Culture Collection – Roscoff Marine Station

CRYOPRESERVATION OF MARINE MICROALGAE USING A CONTROLLED RATE FREEZER

MATERIALS & REAGENTS

Microalgal culture

Ethanol for sterilisation

Pipettes /Pipette tips

Labelled 1.8ml cryovials

Cryobox

-150°C freezer or Liquid Nitrogen storage facility

50ml tubes for mixing cryoprotectant

Controlled rate freezer

DMSO

Sterile culture medium

Sterile filter-cap culture flasks

Syringe & 0.2 µm filter

Large Plastic/ metal forceps

Inverted microscope

Label printer

Labels

PPE for handling Liquid nitrogen

Health and safety rules:

- Any operator using or transporting liquid nitrogen should be well protected: lab coat, gloves and protective glasses OBLIGATORY AT ALL TIMES.
- When using DMSO, standard security measures for handling of chemical reagents should be respected.
- Throughout the protocol, all consumables (cryotubes, flasks, Falcon tubes, tips…) coming into contact with the strain must subsequently be put in an autoclave bag and autoclaved (120°C, 20 mins) prior to disposal.

Preparation of cultures

- Strains are typically cryopreserved towards in mid- to late-exponential phase of batch culture growth (i.e. relatively dense, actively growing cultures).
- The purity and healthy status of strains should systematically be verified by observation under a light microscope prior to cryopreservation.
- Cell density can be quantified prior to cryopreservation by flow cytometry and/or light microscope counting chamber.

Preparation of cryotubes

- Make a 20% or 10% v/v solution of dimethyl sulfoxide (DMSO) in sterile seawater and filter-sterilize this solution using a 0.2 micron pore size syringe filter.
- Mix 10ml of the DMSO solution with 10ml of culture in a sterile Falcon tube, then distribute 1ml of the culture/DMSO mix into each of 10 labelled cryotubes.

Cryopreservation protocol

- 10 minutes after mixing the DMSO and culture, put cryotubes into rods, place rods in the controlled rate freezer, and run the following programme:
    - Begin at the same temperature as the original culture
    - Decrease temperature by 1°C/min until -40°C
    - Hold at -40°C for 10 minutes
- When the cycle is finished, remove rods from controlled rate freezer, remove cryotubes from rods and plunge cryotubes into liquid nitrogen (in isotherm container / polystyrene box).
- Transfer cryotubes to a labelled cryobox in the liquid nitrogen container and/or -150°C freezer.

MICROALGAE CRYOPRESERVATION PROTOCOL

L. Gouhier, P. Gourvil, I. Probert

Roscoff Culture Collection – Roscoff Marine Station

CRYOPRESERVATION OF MARINE MICROALGAE USING A PASSIVE FREEZING DEVICE

Health and safety rules:

- Any operator using or transporting liquid nitrogen should be well protected: lab coat, gloves and protective glasses OBLIGATORY AT ALL TIMES.
- When using DMSO, standard security measures for handling of chemical reagents should be respected.
- Throughout the protocol, all consumables (cryotubes, flasks, Falcon tubes, tips…) coming into contact with the strain must subsequently be put in an autoclave bag and autoclaved (120°C, 20 mins) prior to disposal.

Preparation of cultures

- Strains are typically cryopreserved towards in mid- to late-exponential phase of batch culture growth (i.e. relatively dense, actively growing cultures).
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- Mix 10ml of the DMSO solution with 10ml of culture in a sterile Falcon tube, then distribute 1ml of the culture/DMSO mix into each of 10 labelled cryotubes.

Cryopreservation protocol

- 10 minutes after mixing the DMSO and culture, put cryotubes into Mr Frosty passive freezing container (filled to the specified level with isopropanol)
- Place Mr Frosty container into -80°C freezer
- After *at least* 2 hours, remove Mr Frosty container from -80°C freezer, remove cryotubes from container and plunge cryotubes into liquid nitrogen (in isotherm container / polystyrene box).
- Transfer cryotubes to a labelled cryobox in the liquid nitrogen container and/or -150°C freezer.

MATERIALS & REAGENTS

Microalgal culture

Ethanol for sterilisation

Pipettes /Pipette tips

Labelled 1.8ml cryovials

Cryobox

-150°C freezer or Liquid Nitrogen storage facility

50ml tubes for mixing cryoprotectant

Mr. Frosty passive freezing container

Isopropanol

DMSO

Sterile culture medium

Sterile filter-cap culture flasks

Syringe & 0.2 µm filter

Large Plastic/ metal forceps

Inverted microscope

Label printer / Labels

PPE for handling Liquid nitrogen

L. Gouhier, P. Gourvil, I. Probert

Roscoff Culture Collection – Roscoff Marine Station

THAWING OF CRYOPRESERVED MARINE MICROALGAE

MATERIALS & REAGENTS

Labelled 1.8ml cryovials
Cryobox
Ethanol for sterilisation
Pipettes / Pipette tips
-150°C freezer or Liquid Nitrogen storage facility
Sterile culture medium
Sterile filter-cap culture flasks
Aluminium foil
Large Plastic/ metal forceps
Inverted microscope
PPE for handling Liquid nitrogen
Water bath
Isotherm container

Health and safety rules:

- Any operator using or transporting liquid nitrogen should be well protected: lab coat, gloves and protective glasses OBLIGATORY AT ALL TIMES.
- When using DMSO, standard security measures for handling of chemical reagents should be respected.
- Throughout the protocol, all consumables (cryotubes, flasks, Falcon tubes, tips…) coming into contact with the strain must subsequently be put in an autoclave bag and autoclaved (120°C, 20 mins) prior to disposal.

Preparation of flasks:

- Prepare flasks with 20mL of the appropriate sterile culture medium for the strain (carefully label the flask; in order to be consistent, use the same label as the cryotube).
- Cover flasks with aluminium foil such that no light can enter the flask.
- Heat the water bath to 25-30°C.

Thawing protocol:

Protect yourself: lab coat, gloves, glasses; ⚠ cryotubes can explode)

- When transporting frozen cryotubes between the liquid nitrogen container or -150°C freezer and the water bath, keep the cryotubes in liquid nitrogen using an isotherm container or polystyrene box.
- Plunge cryotubes into the water bath.
- Remove the cryotube from the water bath as soon as there is no more ice in the cryotube (visual inspection) – this usually takes 2 to 3 minutes.
- Thoroughly wipe the cryotube with 70% ethanol. Proceed to the next step immediately.

Transfer of samples:

- Under a laminar flow hood pour the contents of the cryotube (approximately 1mL) into the designated flask (covered with aluminium) containing 20mL of the appropriate culture medium.
- Remove the labels from the cryotube and stick one on the flask.
- Put the flasks at the same temperature as the original culture.
- After 24 hours, remove the aluminium foil from the flask and expose them to the same light regime (and temperature) as the original culture.

Surveillance of samples

- Every 2 days visually check the flask for colour and/or observe under a light microscope and/or analyse by flow cytometry (*remark* : it can take a long time – 1 to 2 months - for cultures to become fully re-established).

CRYOPRESERVATION OF HYPERSALINE DUNALIELLA STRAINS PROTOCOL

A. Ward

The Marine Biological Association of the UK – Plymouth, UK

Cryopreservation of hyper saline Dunaliella salina, minuta, tertiolecta, velox, viridis and bioculata.

1. The cultures to be cryopreserved should be healthy, free from contamination and in the late exponential growth phase. This growth phase can be determined by daily cell counts in a time preceding cryopreservation.

2. These strains should be cryopreserved in the same media in which they are grown. For this species, the medium F/2 + 50g L-1 Sea salts is recommended. All media used should be sterilised by autoclave or filter sterilisation 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 sterilised and pipettes tips have filters to avoid any contamination from the pipette.

3. The hypersaline Dunaliella strains outlined above can be cryopreserved in 10% (v/v) Dimethyl Sulphoxide (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 sterilised using a 0.22µm 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 should be allowed for the solution to return to room temperature before adding to the cell culture.

MATERIALS & REAGENTS

Dunaliella cultures

Ethanol for sterilisation

Pipettes

Pipette tips

Labelled 1.8ml cryovials

Cryobox

-150°C freezer or Liquid Nitrogen storage facility

50ml tubes for mixing cryoprotectant

Passive freezing container

DMSO

L1 Culture media

Syringe & 0.2 µm filter

Large Plastic/ metal forceps

Microscope

PPE for handling Liquid nitrogen

4. 1ml of this culture/cryoprotectant mixture is then aliquoted into 1.8ml cryogenic vials and placed into a passive freezing container commonly known as a 'Mr Frosty'. This is a container that is filled with isopropanol and has a section to place the cryovials so they are surrounded by, but not immersed in the isopropanol. This container achieves a freezing rate of 1°C min^{-1}. (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 minutes in

order for the cryoprotectant to penetrate the cells. The passive freezing container is then placed into a -80°C freezer for 100 minutes.

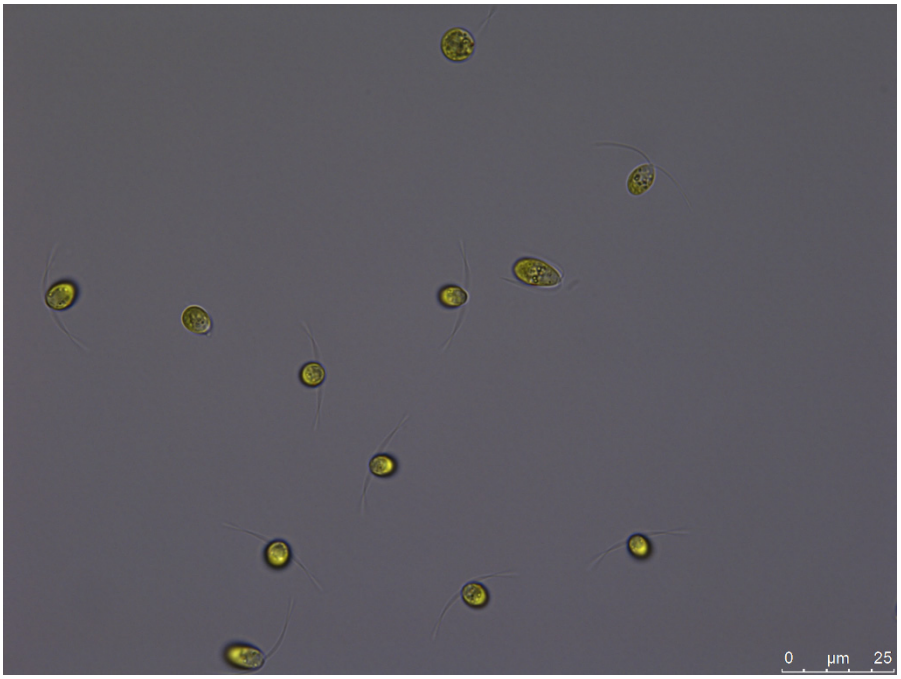

Figure 1.- Healthy Dunaliella culture

5. After this time the container is removed and the cryovials plunged individually into Liquid Nitrogenusing large forceps and the correct 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 37-40°C water bath until all ice crystals are gone (no longer than 2 minutes) . It is important to note that other vials that are stored alongside the one to be thawed shouldn't 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 (eg: cryobox) can be placed into liquid nitrogen while the vials to be thawed are removed. 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 20ml 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 hours. After this time the lid is gradually opened to allow some light in. After 48 hours 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 six weeks for a strong culture to establish.

9. A healthy viable culture is one that is free from contamination and where the cells are of similar morphology and motility as before the cryopreservation procedure.

BIBLIOGRAPHY FOR FURTHER CONSULTATION:

1.-Rhodes, L., Smith, J., Tervit, R., Roberts, R., Adamson, J., Adams, S., Decker, M. Cryopreservation of economically valuable marine micro-algae in the classes Bacillariophyceae, Chlorophyceae, Cyanophyceae, Dinophyceae, Haptophyceae, Prasinophyceae, and Rhodophyceae. Cryobiology 52, (2006), 152–156 10.1016/j.cryobiol.2005.10.003

2.-Ben-Amotz, A., Jürgen, E., Polle, W., Subba Rao, D.V. (2009) The Alga Dunaliella, Taylor & Francis

CRYOPRESERVATION OF AMPHIDINIUM CARTERAE PROTOCOL

A. Ward

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MATERIALS & REAGENTS

- *Amphidinium carterae culture*
- *Ethanol for sterilisation*
- *Pipettes*
- *Pipette tips*
- *Labelled 1.8ml cryovials*
- *Cryobox*
- *-150°C freezer or Liquid Nitrogen storage facility*
- *50ml tubes for mixing cryoprotectant*
- *Passive freezing container*
- *DMSO*
- *L1 Culture media*
- *Syringe & 0.2 µm filter*
- *Large Plastic/ metal forceps*
- *Microscope*
- *PPE for handling Liquid nitrogen*

1. The cultures to be cryopreserved should be healthy, free from contamination and in the late expontential growth phase. This growth phase can be determined by daily cell counts in a time preceding cryopreservation.

2. These strains should be cryopreserved in the same media in which they are grown. For this species L1 media is recommended. All media used should be sterilised by autoclave or filter sterilisation 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 sterilised and pipettes tips have filters to avoid any contamination from the pipette.

3. *Amphidinium carterae* can be cryopreserved in 10% (v/v) Dimethyl Sulphoxide (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 sterilised using a 0.22µm 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 should be allowed for the solution to return to room temperature before adding to the cell culture.

4. 1ml of this culture/cryoprotectant mixture is then aliquoted into 1.8ml cryogenic vials and placed into a passive freezing container commonly known as a 'Mr Frosty'. This is a container that is filled with isopropanol and has a section to place the cryovials so they are surrounded by, but not immersed in the isopropanol. This container achieves a freezing rate of 1°C min^{-1}. (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 minutes in order for the cryoprotectant to penetrate the cells. The passive freezing container is then placed into

a -80°C freezer for 100 minutes.

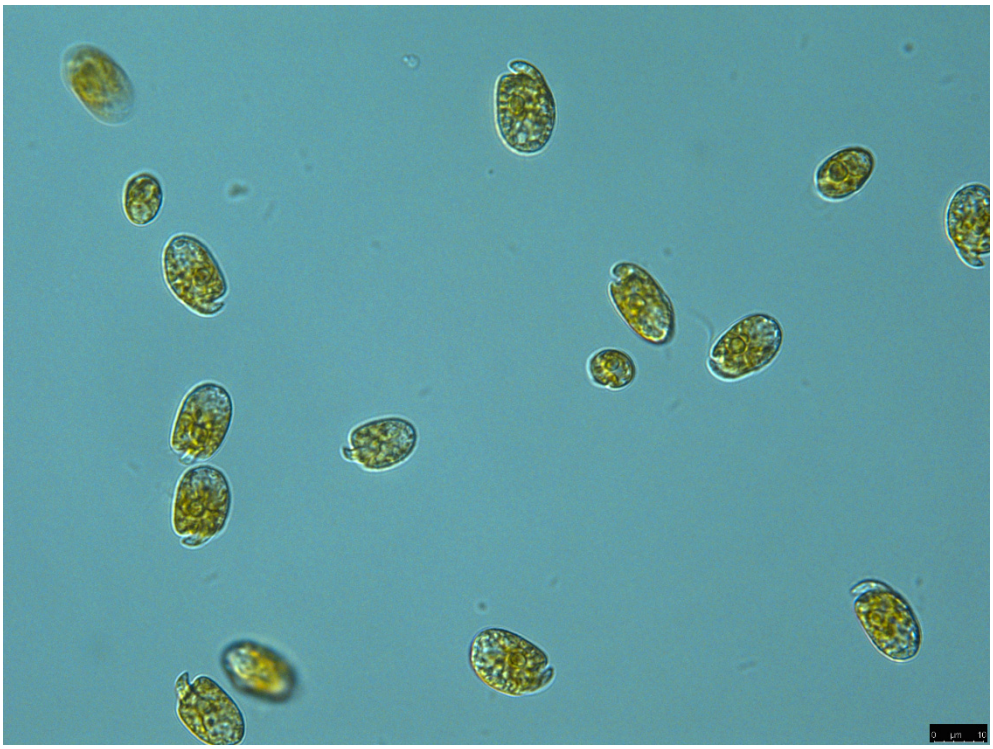

Figure 1.- Healthy Amphidinium culture

5. After this time the container is removed and the cryovials plunged individually into Liquid Nitrogenusing large forceps and the correct 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 37-40°C water bath until all ice crystals are gone (no longer than 2 minutes) . It is important to note that other vials that are stored alongside the one to be thawed shouldn't 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 (eg: cryobox) can be placed into liquid nitrogen while the vials to be thawed are removed. 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 20ml 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 hours. After this time the lid is gradually opened to allow some light in. After 48 hours 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 six weeks for a strong culture to establish.

9. A healthy viable culture is one that is free from contamination and where the cells are of similar morphology and motility as before the cryopreservation procedure.

BIBLIOGRAPHY FOR FURTHER CONSULTATION:

1.-Rhodes, L., Smith, J., Tervit, R., Roberts, R., Adamson, J., Adams, S.L., Decker, M. Cryopreservation of economically valuable marine micro-algae in the classes Bacillariophyceae, Chlorophyceae, Cyanophyceae, Dinophyceae, Haptophyceae, Prasinophyceae, and Rhodophyceae. Cryobiology 52 (2006), 152–156 10.1016/j.cryobiol.2005.10.003

2.-Hulburt, E.M. The taxonomy of the unarmoured dinoflagellates of shallow embayments on Cape Cod, Massachusetts. Biol. Bull., 112 (1957), 196-219. 10.2307/1539198

MICROALGAE ENCAPSULATION-VITRIFICATION

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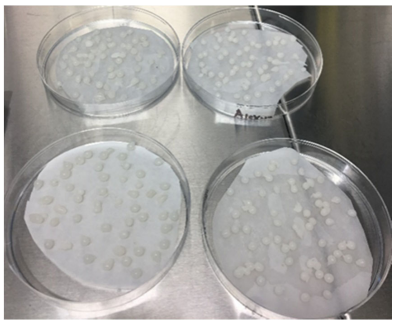
ENCAPSULATION/DEHYDRATION- VITRIFICATION

1. Mix the algae (concentration 10^5 for larger algae to 10^7 for smaller algae) with the sodium alginate mixture (**solution 1)** and Sucrose (**Solution 4**).
2. Then drop gently with a Pasteur pipette/syringe e into the $CaCl_2$ (**Solution 2**) to form the beds and allow the beads to equilibrate 60 minutes.
3. Transfer the beads to a sterile petri dish to dry. Let them dry for an hour in a laminar air-flow chamber over filter paper. Then take the beads into cryovials, mix them with the cryoprotectant (**solution 5**). After 15 minutes plunge the cryovials into liquid nitrogen.
4. To thaw the cryovials plunge them into 35ºC water for 2 minutes.
5. Then take the beads into a 0, 3 M sodium citrate (**solution 3**). Allow the beads to dissolve for 60 minutes.
6. Then take the solution into fresh culture medium and put it into semi dark conditions for 24 hours.
7. Elapsed that time observe the cells under the microscope for qualitative viability assessment /cell fitness.
8. Incubate in normal culture conditions.



MATERIALS & REAGENTS

(1) **Sodium Alginate:** *(sodium alginate 7 g + culture medium 100ml) and shake gently.*

*(2)***$CaCl_2$ 100mM in culture media:** *CaCl2 11,09 g + distilled water 1 L) into a long test tube*

(3) **Sodium Citrate 0.3M:** *Sodium citrate 1,54 g + culture medium 20 ml*

(4) **Sucrose 0.5M**

(5) **Cryoprotectant** *selected in culture media*

QUALITY CHECK POINTS POST VITRIFICATION:

Cell density/time

Cell fitness (morphological exam, motility, division)

Fluorescence in vivo

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.- Amaral, R., Santos, M.F., Santos., L.M.A Overcoming recalcitrance in *Porphyridium aerugineum* geitler employing encapsulation-dehydration cryopreservation methods. CryoLetters 30 (2009), 462-472

2.-Paredes, E., Costas, D., Casal, A., Cortina-Burgueño, A., Lubián ,L.M. Criopreservación de microalgas marinas: *Nannochloropsis gaditana, Rhodomonas lens, Cylindrotheca closterium, Chaetoceros gracilis, Synechoccocus sp. e Isochrysis aff. galbana clon T-ISO. V Foro Iberoam. Rec. Mar. Acui. Cárdenas S., Mancera J.M., Rey-Méndez M., Lodeiros C. (eds.):263-273.* 10.13140/RG.2.1.5036.8726

3.-Chen, Y. Immobilized *Isochrysis galbana* (Haptophyta) for long-term storage and applications for feed and water quality control in clam (*Meretrix lusoria*) cultures. *Journal of Applied Phycology* 15 (2003), 439–444 10.1023/A:1026071714199

IMPORTANCE OF FISH SPERM CRYOPRESERVATION

Zebrafish, Sole and Grey mullet

Cryopreservation is of interest not only for fish farming but also for the conservation and genetic improvement of resources. Germplasm cryobanking has important applications in reproductive practices in cultured marine and freshwater aquatic species by simplifying broodstock management through synchronization of gamete availability or even by allowing the safety transportation of gametes. Its potential has also been evident in maintaining important strains of laboratory model fish species, such as zebrafish or medaka. Cryobanking has also been a valuable tool to preserve the genetic resources of a wide range of species and with the help of reproductive biotechnologies, such as germ cell xenotransplantation, it plays an important role in genetic selection programs, biodiversity preservation and assisted reproduction. Cryopreservation could be a secure method to preserve species genetic material, providing the opportunity to preserve representative samples and further reconstruct the original strain, population or diversity. In conclusion the use of cryobanks would certainly benefit the aquaculture industry, reinforcing the tremendous impact that biotechnology is having in aquaculture, in model species for medical research and in conservation of wild genome.

The management of these banks requires technical capacity in genetics, reproductive physiology, cryobiology and data administration.

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References:

Adams, S.L., Smith, J.F., Tervit, H.R., McGowan, L.T., Roberts, R.D., Achim, R.J., King, M.G., Gale, S.L. Webb, S.C. Cryopreservation of molluscan sperm: oyster (Crassostrea gigas, Thunberg), mussel (Perna canaliculus) and abalone (Haliotis iris). In: Cryopreservation in Aquatic Species, 2nd Edition. T. R. Tiersch and C. C. Green (Eds.) World Aquaculture Society (2011) Baton Rouge, Louisiana, pp: 562-573.

Paredes, E. Exploring the evolution of marine invertebrate cryopreservation - landmarks, state of the art and future lines of research. Cryobiology 71 (2015), 198-209. 10.1016/j.cryobiol.2015.08.011

Suquet, M., Labbé, C., Puyo, S., Mingant, C., Quittet, B., Boulais, M., Queau, I., Ratiskol, D., Diss, B., Haffray, P. Survival, growth and reproduction of cryopreserved larvae from a marine invertebrate, the Pacific oyster (Crassostrea gigas). PLoS ONE 9, 4 (2014), 0093486. doi.org/10.1371/journal.pone.0093486

Zhang, T. Cryopreservation of gametes and embryos of aquatic species. In: Life in the frozen state. B.J. Fuller, N. Lane, E.E. Benson (Eds.) CRC Press (2004), 415-136.

Zefrafish Sperm Collection

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MATERIALS & REAGENTS

Electric ultrafreezer (-150 ºC)

Cooling rate (-66 ºC/min)

Cryovials 2ml

Liquid nitrogen

HBSS:

(NaCl 8.0 g, KCl 0.4 g, $CaCl_2$ x $2H_2O$ 0.16 g, $MgSO_4$ x $7H_2O$ 0.2 g, Na_2HPO_4 0.06 g, KH_2PO_4 0.06 g, $NaHCO_3$ 0.35 g, $C_6H_{12}O_6$ 1.0 g in 1000 mL milli-Q water, pH 7.5)

Tricaine:

400 mg tricaine powder, 97.9 ml mili-Q water, 2.1 ml 1 M Tris (pH 9).

Freezing solution: *HBSS with 20% N-N dimethylformamide (DMF)*

QUALITY CHECK OF THE GAMETES:

Sperm motility and plasma membrane viability

Sperm: >50 % motile

ZEBRAFISH SPERM COLLECTION

1- Select zebrafish males with 6-8 months of age that are maintained separated from females in the tanks to avoid uncontrolled gametes release.
2- On the day prior to the sperm collection, place males and females in 1 L breeding tanks (Tecniplast, Buguggiate, Italy) maintained both sexes separated while sharing the same water for 16 h.
3- Collect sperm within 1 h after the beginning of the light phase of the photoperiod.
4- Anesthetize males with 0.168 mg/ml tricaine sulfonate solution (MS-222, Sigma-Aldrich). When the gill movement decrease. Rinse the males with Phosphate Buffered Saline (PBS) solution and carefully clean their body with paper towels.
5- Collect sperm through abdominal massage using a glass capillary tube attached to a mouth piece.
6- Dilute sperm immediately after collection into 10 µl of sterilized and filtered (0.20 µm) Hank´s Balanced Salt Solution (HBSS).

SPERM CRYOPRESERVATION PROCEDURE

SPERM CRYOPRESERVATION:

1- Select sperm samples with total motility over 50% (at 10 s post activation) and cell concentration over 3 x 10^7 cells/mL. Sperm pools can be performed if necessary.
2- Cryopreserve zebrafish sperm in cryovials adding pre-diluted sperm to the freezing media 1:1 (v:v) in a total volume of 10 µl, with a final concentration of 10% of DMF, with 3 minutes of equilibration.

3- Perform a cooling rate of -66 ºC/min placing the cryovials with sperm diluted in the freezing medium directly in an electric ultrafreezer (-150 ºC), performing the cooling rate and sample storage simultaneously.
4- Thaw the frozen cryovials in a water bath set at 40°C for 8 s.
5- Analyse sperm quality immediately after thawing since the viability of spermatozoa decreases with post-thaw time.

SPERM QUALITY ANALYSIS

SPERM MOTILITY ASSESSMENT

1- Use CASA system settings set for 25 frames per second to evaluate sperm motility.
2- Add 1 µl of pre-diluted sperm on the Makler chamber.
3- Activate sperm motility with 5 µl of filtered and sterilized system water (700 µS/cm) set at 28 ºC.
4- Record motility parameters during one minute in 4-6 time-points post activation.

SPERM VIABILITY ASSESSMENT

1- For flow cytometer analysis, dilute 5 µl of SYBR 14 stock solution in 120 µl of sterilized and filtered HBSS.
2- In the flow cytometer tube add 2 µl of sperm in 300 µl of HBSS.
3- Label the cells with 1 µl propidium iodide (PI) at a concentration of 2.4 mM and 0.5 µl of pre-diluted SYBR 14.
4- Analyse in a flow cytometer after 5 min of incubation in the dark.

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SPERM COLLECTION AND STORAGE OF *Solea senegalensis* SPERM

1- Anesthetize males with 300 ppm 2-phenoxyethanol for 10 min before sperm collection.

2- Dry out urogenital pore with paper towel and collect the sperm using a syringe (without needle) or a 20 µl micropipette by gently pressing the testes.

3- Store the samples in eppendorfs in a styrofoam rack on ice until further analysis. Discard samples contaminated with water, urine or faeces.

4- If samples need to be transported, centrifuge sperm and discard seminal plasma to eliminate possible urine contamination. Re-dilute sperm cells into Ringer solution (equivalent to the initial seminal plasma volume).

SPERM CRYOPRESERVATION PROCEDURE

SPERM CRYOPRESERVATION:

1- Add sperm to the freezing solution 1:2 (v:v) and load into 0.25 ml French straws with 2 min of equilibration.

2- Place the straws 2 cm above liquid nitrogen in a styrofoam box to perform a cooling rate for 10 minutes. Plunge the samples into liquid nitrogen and store in the cryobank.

3- Thaw the straws in a water bath set at 25°C for 10 s.

4- Perform the sperm quality analysis immediately after thawing, since the viability of spermatozoa decreases with post-thaw time.

MATERIALS & REAGENTS

Styrofoam box (suspended 2 cm above liquid nitrogen)

French straws 0.25 ml

Freezing solution: Mounib solution (Sucrose 125 mM, $KHCO_3$ 100 mM , reduced glutathione 6.5 mM) with 10% DMSO and 10% egg yolk.

Non-activating medium: Ringer solution (HEPES 20 mM, KH2PO4 5 mM, MgSO4 1 mM, CaCl2 1 mM, NaCl 136 mM and KCl 4.7 mM)

Liquid nitrogen

Sperm motility activation solution:

Seawater (SW) at 21°C and 35 ppt salinity

QUALITY CHECK OF THE GAMETES:

Sperm motility and plasma membrane viability analysis

Average sperm volume of F0 males have a range of 10-150 µl while F1 males (5-50 µl)

Sperm: > 75 % motile

SPERM QUALITY ANALYSIS

Prior quality analysis, sperm is diluted 1:9 in a non-activating medium (Ringer solution).

SPERM MOTILITY ASSESSMENT

1- Use CASA system settings set for 50 frames per second to evaluate sperm motility.
2- Add 1 µl of pre-diluted sperm to the Makler chamber.
3- Activate sperm motility with 5 µl of artificial seawater
4- Record motility parameters during one minute (each 15 s) post activation.

SPERM VIABILITY ASSESSMENT

1- Dilute 5 µl of sperm in 500 µl of 1% NaCl buffer.
2- Add 2.5 µl of propidium iodide PI at a final concentration of 1 µl/ml.
3- Incubate during 5 min in the dark.
4- Analyse in a flow cytometer.

I. Cancio

Plentzia Marine Station (PiE-UPV/EHU) – University of the Basque Country

MATERIALS & REAGENTS

- *Mature male thicklip grey mullets*
- *Falcon tubes and cryovials*
- *Benzocaine*
- *PBS and VZE extender*
- *DMSO*
- *Liquid Nitrogen (LN_2)*
- *Filtered Sea Water*
- *Microscope coupled to CASA software*

QUALITY CHECK OF THE SPERM:

Motility of the sperm is analysed under the polarised microscope and mobility parameters analysed with the CASA softwares

Mugilid fish species are widely distributed in coastal waters. The thicklip grey mullet *Chelon labrosus* is a eurihalyne species that inhabits estuarine waters during most of the year, migrating to the ocean for spawning and reproduction. Larvae return soon to estuarine areas where they grow. Exposure to xenoestrogens in continental waters provokes the development of the intersex condition in males. This is a feminisation process by which oocytes are developed inside testis (Fig. 1) and it has been widely reported in the Basque estuaries (Ortiz-Zarragoitia et al., 2014). Analysing the quality of the sperm of such xenoestrogen exposed mullets is relevant in the assessment of the health of the mullet population, and in environment quality assessment.

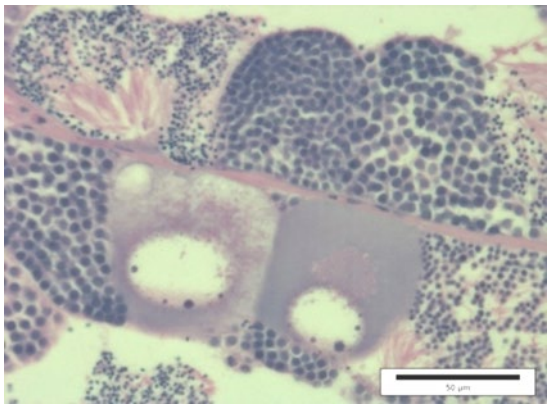

Figure 1. – Histological cut of *Chelon labrosus* testis with presence of oocytes

MULLET SPAWNING BY GENTLE MASSAGE

1. Mullets begin to be ripe around December and until early February that is when they initiate reproductive migration.
2. Fish mullets inside estuaries in between December-February.
3. Place mullets in a bath saturated with benzocaine (50 ppm of Benzocaine) to anesthetize them.

4. Dry the ventral part of the fish and gentle massage with the thumb and forefinger toward the urogenital opening from where sperm will directly pour into a falcon tube (Fig. 2)
5. Within estuaries spawn is very dense (sperm is possibly finally hydrated in the sea) and needs to be diluted in PBS 1/5.

Figure 2. - Thicklip spawning after massage

6. Dilute a ratio of 1 part of sperm and 1 of cryomedium using V2E extender (128,5 mM NaCl, 5,10 mM KCl, 24 mM NaHCO·, 1 mM glucose, egg yolk 0,1 ml, pH 8,2) and DMSO at a final concentration of 10% in 2 ml cryotubes.
7. Equilibrate 5 to 10 min at room temperature and freeze using a one-step freezing protocol at -20ºC/min from +4 to -150ºC and then plunge into LN_2, (better than direct plunging into LN_2).
8. Store frozen sperm in LN2 and thaw by gentle agitation in a water bath at 30ºC for 90-120 seconds.
9. Analyse mobility under the microscope using marine water to activate sperm motility (Fig. 3)

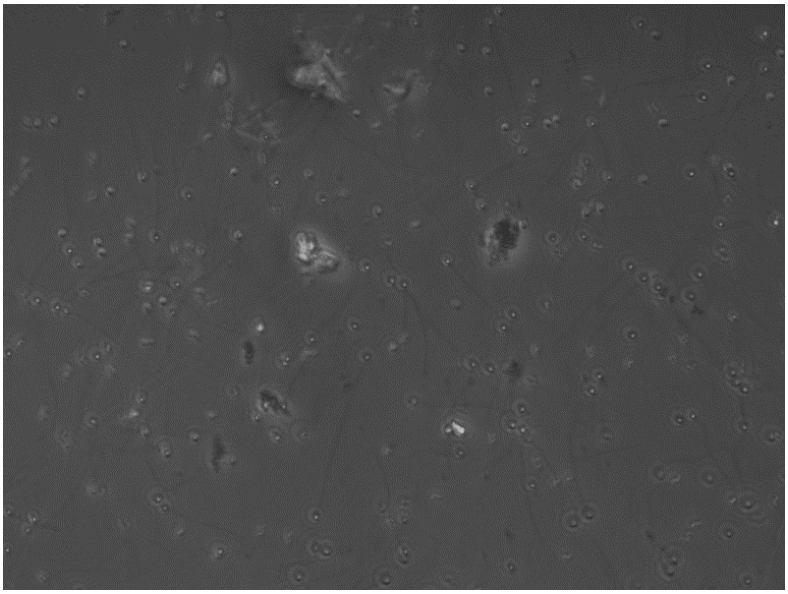

Figure 3.- Thicklip grey mullet sperm moving as captured while measuring mobility with the CASA program

BIBLIOGRAPHY FOR FURTHER CONSULT:

1.-Ortiz-Zarragoitia, M. Diaz de Cerio, O; Rojo-Bartolomé, I; Bizarro, C; Cajaraville, M.P. & Cancio, I. Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine waters. Marine Drugs 12 (2014), 4756-4782. doi.org/10.3390/md12094756

2.-Balamarugan, R; Prapapron, W; Munuswamy, N. Sperm activation and effects of cryopreservation on motility, ultrastructure and DNA integrity in Grey mullet Mugil cephalus. Aquaculture Report 14, (2019) 100204 doi.org/10.1016/j.aqrep.2019.100204

3.-Balamarugan, R; Munuswamy, N. Assessment of Sperm quality and short term preservation of sperm in Grey mullet Mugil cephalus (Linnaeus, 1758). Indian Journal of Fisheries 63,(2016), 140-144. 10.21077/ijf.2016.63.4.61001-23

CRYOPRESERVATION OF MICROBIAL MATS

Cross section of a coastal microbial mat from the Dutch barrier island of Schiermonnikoog

Coastal and hypersaline microbial mats are a special type of biofilm consisting of bacteria, archaea and micro-eukaryotes. Through the extrusion of extracellular polymeric substances (EPS) these organisms form a dense matrix with the sediment particles in a structure that resembles a mat, hence microbial mat. They form approximately 5 mm thin, nearly closed self-sustaining minimal ecosystems with complete element cycles of carbon, nitrogen, phosphate and sulfur and are driven by photosynthetic primary producers, mainly cyanobacteria and diatoms. They form characteristic colored layers along a vertical geochemical gradient of light, oxygen and sulfide with green photosynthetic microbes on top, a purple layer of sulfide oxidizing bacteria followed by a black anoxic later in which sulfate reducers may reside. Despite its simple appearance, molecular analysis of coastal microbial mats revealed a complex, diverse community with up to 1000 different microbial species.

They form ideal model systems for the analysis of interspecies interactions and evolution of complex communities. In a sense, these mats are 5 mm deep analogs of the open ocean with a photic and aphotic zone, with oxic and anoxic zones, and with different representatives of a food web ranging from primary producers, decomposers, grazers and viruses. Microbial mats are also considered as modern equivalents of Precambrian stromatolites, the oldest ecosystems known from the fossil record and are used as model systems for terraforming in astrobiology. Moreover, their tight physical coupling make them ideal model systems for synergistic interactions and studies towards understanding the formation of multicellular life. Finally, they are model systems for how cyanobacterial circadian rhythms may drive rhythmic metabolic networks in coastal microbial mats.

Microbial mats have several important applications. Stabilization of coastal sediments by microbial mats occur through the production of copious amounts of EPS. In addition, the mats enrichment the sediment with organic carbon and nutrients thereby allowing the colonization of the normal infertile loose beach sand with a salt marsh vegetation. This process completely transformed the North Sea beach of the Dutch barrier island Schiermonnikoog over the past 30 years from a long stretched sandy beach into a broad salt marsh covering large areas of the island's North Sea coast; an excellent example of how microbes influence coastal morphodynamics and protection. Initial experiments also show an important contribution of microbial mats in the bioremediation of crude oil and plastics and we currently investigate the usage of the salt marsh microbiome in facilitating saline agriculture of potatoes and halophytic crops. Although several types of microbial mat are easily accessible and nearby research facilities, other types of mats and complex microbial communities are less accessible because of their location. For example, sediment samples from the deeper parts of the oceans or other remote places on earth are very costly in their acquisition and are prone to rapid community changes once extracted from their environment. A quick and good freezing and cryo-preservation protocol allows for long-term preservation of intact samples. This is also of importance for more accessible samples to which one wish to make different types of analysis in the present time but also in the future. Biobanking of precious cryo-preserved samples will be essential for past and future reference and can be re-analyzed when novel techniques become available or when initial analysis detect the presence of organisms of biotechnological or pharmaceutical importance.

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REFERENCES:

Bolhuis, H., Stal, L.J. Analysis of bacterial and archaeal diversity in coastal microbial mats using massive parallel 16S rRNA gene tag sequencing. Isme Journal 5 (2011), 1701-1712. 10.1038/ismej.2011.52

Stal, L.J., Bolhuis, H., Cretoiu, M.S. Phototrophic marine benthic microbiomes: the ecophysiology of these biological entities. Environmental Microbiology 21(2019), 1529-1551. 10.1111/1462-2920.14494

Cryopreservation of Microbial Mats

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CRYOPRESERVATION PROTOCOL FOR MICROBIAL MATS

1. 350 ml of growth medium was added to 50 gram of hypersaline microbial mat (Guerande, France) and blended to create a homogeneous slurry.
2. Distribute the slurry over 35 sterile plastic 15 ml tubes (8 ml slurry per tube) and add 2 ml of cryoprotective agent (CPA) at a final concentration of Methanol (5% v/v), DMSO (10% v/v) and Glycerol (10% v/v) or add 2 ml sterile medium in the non cryopreservative controls (NCPA)).
3. After thorough resuspension, 2 ml of this mixture is transferred to 5 sterile cryo-vials per sample (or more depending on need).
4. Samples were slowly frozen (-1ºC/min - 4 hrs) until -80ºC using “Mister Frosty” and then placed in a -150ºC freezer.

TESTING CRYOPRESERVATION EFFICIENCY

1. Fill 150 ml glasses with 100 grams of washed beach sand; add 50 ml hypersaline medium. Put the glass inside the plastic cultivation box, add 50 ml millliQ water outside the glass to maintain humidity and autoclave 20 min at 121ºC.

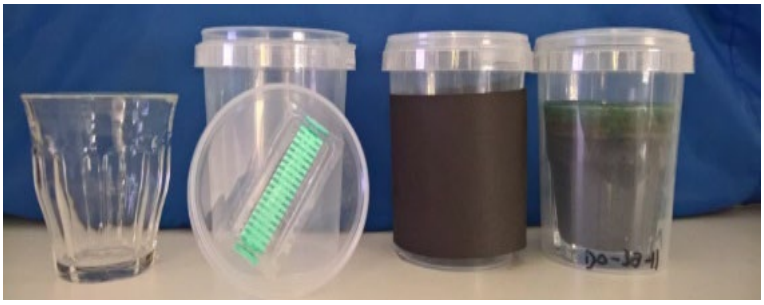


Fig. 1 Experimental set up.

MATERIALS & REAGENTS

Hypersaline microbial mat material

Plastic cultivation Box (SacO2 microbox)

Glass150 ml (IKEA)

Washed beach sand

Hypersaline medium (13% salinity)

MilliQ water

Incubator (37℃)

Freezer (-150°C)

Cryopreservative (Methanol , DMSO, Glycerol)

DNA extraction kit (QIAGEN)

Sterile plastic ware

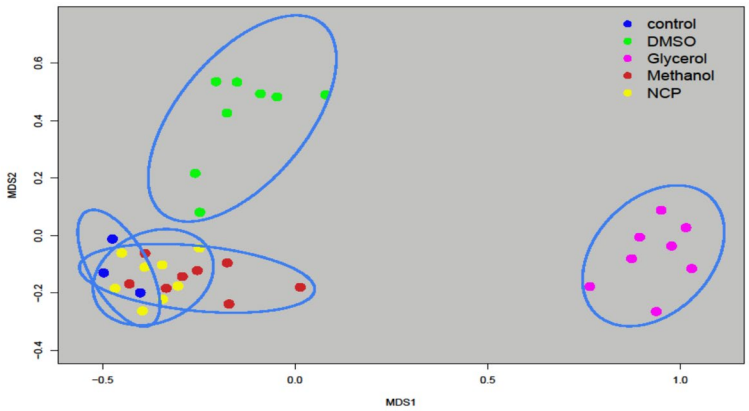
Mister Frosty (Nalgene)

2. Samples were kept at -150ºC for 1, 70 or 129 days, controls that were not frozen (T=0) were immediately used.
3. Regrowth of hypersaline mats was performed by mixing the 2 ml slurries with 8 ml fresh sterile medium and added to the top of the sterile beach sand/medium mixture.
4. Artificial mats were incubated in the dark (aluminium foil covered) at 37ºC for 24 hrs, received some light (holes in the foil) for 48 hrs and then incubated at a 14/10 hr light dark cycle for 120 days.
5. After 120 days, samples from the top 1 cm were taken for DNA extraction and DNA was send out for amplicon sequencing.

INITIAL RESULTS OF EFFECT CRYOPRESERVATION ON BACTERIAL COMMUNITY COMPOSITION.

Based on similarity in bacterial community composition after cryopreservation (Figure below), the best cryoprotective agent is methanol (5% v/v) or absence of an agent (NCPA). Both glycerol and DMSO dramatically change the microbial composition relative to the untreated control community. Especially when glycerol is carried over in the growth medium the community changes dramatically as a result of heterotrophic growth on glycerol as carbon and energy source leading to rapid anoxic and favouring anaerobic bacteria in the 120 days incubation period. Incubation time had also a negative but less significant effect on the community composition relative to the initial natural state.

Although absence of any cryopreservative may seem to give the best results for the bacterial part of the community this is not expected to be the same for micro-eukaryotes. Therefore further investigation and optimization is needed to include the micro-eukaryote community and test different microbial mat samples and communities, which will in part be carried out as deliverable in the H2020 project SIMBA (https://simbaproject.eu/). The combined results will be published as a peer reviewed scientific publication (Fig. 2 below).



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