



Short communication

Non-cryogenic preservation of thalli, germlings, and gametes of the green seaweed *Ulva rigida*Guang Gao ^{a,1}, Anthony S. Clare ^a, Craig Rose ^b, Gary S. Caldwell ^{a,*}^a School of Marine Science and Technology, Ridley Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, England, UK^b Seaweed & Co. Ltd, Office 2.3 North Tyneside Business Centre, 54a Saville Street, North Tyneside, NE30 1NT, England, UK

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ABSTRACT

There is a need to develop reliable and cost-effective preservation techniques that support the growing demand for *Ulva* cultivation. Using combinations of two dimethyl sulfoxide concentrations (DMSO; 10 and 15%), three non-cryogenic temperatures (4, -20 and -80 °C), and one- and two-step cooling, we evaluated the effectiveness of seven methods for preserving *Ulva* thalli, germlings, and gametes for periods of up to 184 days. Preservation success was assessed using post-thaw regrowth and a pigmentation index. Refrigeration at 4 °C without DMSO successfully preserved thalli for 184 days with little loss of plant quality. In contrast, the freezing methods produced low regrowth success and poor quality thallus tissue, with few plants surviving beyond 30 storage days. Freezing temperatures were lethal to germlings after storage for only one day, but germlings preserved at 4 °C had regrowth rates comparable to thalli preserved under the same conditions. In contrast, *Ulva* gametes were successfully preserved at freezing temperatures for 184 days although viability was relatively low (7.0–18.7% at -20 °C and 3.5–12.1% at -80 °C). Gamete viability at 4 °C decreased from 94.74 ± 4.30% to 26.12 ± 3.97% when the storage time was extended from one to 184 days. DMSO (10%) reduced gamete viability both at -20 °C and -80 °C. We demonstrate for the first time, the contrasting responses of *Ulva* thalli, germlings, and gametes to different non-cryogenic preservation methods.

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1. Introduction

Having the capacity to reliably preserve live algae tissue and cell lines will advance seaweed cultivation, particularly in a hatchery context. Many microalgae have been successfully cryopreserved and the approach is now commonplace in culture collections (Day, 1998). In comparison, the preservation of seaweeds is less advanced and literature records are few.

Porphyra (both conchocelis and thalli) (Kuwano et al., 1992, 1993, 1994, 1996; Migita, 1964, 1966, 1967), *Undaria* (Arbault et al., 1990; Ginsburger-Vogel et al., 1992; Renard et al., 1992), and *Laminaria* (Vigneron et al., 1997) species have been preserved using two-step methods. Combination of cryoprotectants with a two-step cooling method have produced >60% survival of *Porphyra* conchocelis stored in liquid nitrogen for one day (Kuwano et al., 1993) and 54.6–70.9% for sporothalli (Jo et al., 2003). The viability of gametophytic thalli of *P. yezoensis* and *P. teneru* has exceeded 95% and in some cases 98% (Kuwano et al., 1996). Encapsulation-vitrification has also been used to preserve seaweeds (Wang et al., 2011; Zhuang et al., 2015). Few

studies report seaweed preservation at non-cryogenic temperatures (above -150 °C), e.g. Migita (1966) and Kuwano et al. (1992), with viability being generally low and decreasing with storage time.

As far as *Ulva* is concerned, reports are mainly confined to preserving zoospores at both cryogenic and non-cryogenic temperatures (Bhattarai et al., 2007; Taylor and Fletcher, 1999b) with the best survival rates at 4 °C (1.4–5.8% after ten days storage). With respect to *Ulva* thalli, Van der Meer and Simpson (1984) cryopreserved *U. lactuca* for at least one hour with 100% survival, and Lee and Nam (2016) cryopreserved gametophytic *U. prolifera* thalli for 120 days achieving viabilities of over 90%.

Our understanding of *Ulva* preservation is poor and this may create a bottleneck for future expansion of the industry. This study used different methods to preserve *U. rigida* gametes, germlings and adults in an attempt to define an optimal non-cryogenic preservation method.

2. Materials and methods

Adult vegetative *Ulva rigida* was collected intertidally from Cullercoats, eastern coast of England (55.03° N, 1.43° W) after a spring tide in June 2014. The thalli were punched into 7 mm diameter disks after rinsing in 1 μm filtered seawater. Gametes were obtained by fragmentation (Hiraoka and Enomoto, 1998) and concentrated using a point light source. Some (1×10^6) of these gametes were used to

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produce germlings. After settlement and germination, 387 germlings of 2 mm length were selected for preservation trials.

DMSO concentrations of 10 and 15% were used for thalli and germlings and 5 and 10% for gametes. Single *Ulva* discs or germlings were placed into 1.5 ml freezing ampoules containing DMSO, with 54 ampoules per temperature treatment; nine of which were assessed for viability after every storage period (one, two, seven, 30, 92, and 184 days). DMSO pretreatment was conducted at 20 °C for 20 min. After pretreatment, two batches were immediately transferred to -20 and -80 °C respectively. Another batch was frozen to -20 °C at a cooling rate of -1 °C minute⁻¹ and then immediately transferred to -80 °C. A batch of samples free from cryoprotectant or pretreatment was placed directly into a 4 °C refrigerator in 100 ml conical flasks and the medium was renewed monthly, and 75% ethanol cotton balls were used to remove any bacterial biofilm. Nine *Ulva* discs and germlings without any preservation treatment were set as a control. Gamete preservation was carried out using a similar method with the omission of the two-step cooling. Fifty four ampoules were used for every temperature treatment, nine of which were assessed for viability after every storage period. Each ampoule contained 2.5 × 10⁶ gametes. Nine ampoules without any preservation treatment were set as a control. Cryopreservation with liquid nitrogen was not employed given its cost in long-term preservation and low success rates in preliminary experiments (data not shown).

After each storage period, the samples were thawed by plunging the ampoules into a 37 °C water bath. As soon as the ice had melted, the cryoprotectant was removed and the samples were repeatedly washed with fresh seawater. Afterwards, thalli and germlings were placed in 100 ml conical flasks (one thallus or germling per flask) and cultured at 18 °C, 80 μmol m⁻² s⁻¹ photon light density, with a 16L: 8D photoperiod for six days. The growth medium was natural seawater supplemented with 150 μM nitrate and 7.5 μM phosphate, and renewed every two days. Gametes were placed in Petri dishes in darkness for 24 h to ensure settlement and then incubated under the conditions described above.

Viability was assessed using two methods; a pigmentation index (PI) ([Van der Meer and Simpson, 1984](#)), and regrowth success. Part or all of the pigmentation of *Ulva* can be lost due to freezing damage. Regrowth was estimated by recording specific growth rate (SGR) over six days at 18 °C. SGR (%) = [ln (M₂/M₁)]/t × 100, where M₂ is the final mass, M₁ is the initial mass, and t is the number of culture days.

After 14 days of culture at 18 °C, gametes were examined by light microscopy and viability was determined by the level of germination (gametes that had divided). The mean percentage germination over nine fields of view (30–40 gametes per field) was recorded. Viability was expressed as a relative rate in order to eliminate effects of low gamete settlement. V = G_t/G_i × 100, where V is the viability of samples, G_t is the germination rate of treated samples and G_i is the germination rate of the reference treatment.

Results were expressed as means ± standard deviation for continuous data and medians ± interquartile range for categorical data. The effects of preservation time on PI were analysed by Friedman tests with Wilcoxon Signed Rank post hoc tests. Temperature and DMSO effects were analysed using Kruskal-Wallis tests with Mann-Whitney post hoc tests. Regrowth rates were normally distributed (Shapiro-Wilk, P > 0.05) and had equal variances (Levene's test, F = 1.457, P > 0.05 for thalli and F = 1.329, P > 0.05 for germlings). Differences in thallus regrowth were analysed by two-way ANOVA at different time points. The effects of preservation temperature, time and DMSO on thallus regrowth were analysed by three-way ANOVA with Tukey honest significant difference (HSD) excluding that at 4 °C which was analysed by one-way ANOVA with Tukey HSD. Viability of gametes was normally distributed (Shapiro-Wilk, P > 0.05) with equal variances (Levene's test, F = 1.117, P > 0.05). Differences in viability between treatments at different time points were analysed by two-way ANOVA with Tukey HSD with three-way ANOVA used to assess the effects of preservation

temperature, time and DMSO, excluding that at 4 °C. Data were analysed using SPSS v.21.

3. Results

The effect of preservation method on the pigmentation index (PI) is given in [Table 1](#). There was a significant difference in PI between preservation times but patterns were different for each temperature (Friedman test, K > 35.000, df = 5, P < 0.001). The PI of samples preserved at -80 °C reduced to zero (dead) by day seven (Wilcoxon Signed Rank test, Z < -2.700, n = 9, P < 0.05) whereas it was above 6.0 for samples stored at -20 °C and 10 at 4 °C. The PI of samples stored at -20 °C decreased to zero (Wilcoxon Signed Rank test, Z < -2.750, n = 9, P < 0.05) by day 30 while samples stored at 4 °C maintained a high PI by day 184. There were significant differences in PI between preservation temperatures regardless of preservation times (Kruskal-Wallis test, K > 50.557, df = 6, P < 0.001). In contrast, samples stored at -80 °C for one day exhibited an extremely low PI. There were no significant differences between the thalli PIs between the two DMSO concentrations except for samples preserved at -20 °C for one day (Mann-Whitney U test, U = 17.500, Z = -2.136, n = 9, P < 0.05). The 15% DMSO concentration enhanced the PI of thalli preserved at -20 °C for one day ([Table 1](#)). The two-step cooling process to -80 °C did not enhance the PI compared with rapid cooling.

Thallus regrowth data are presented in [Table 2](#); treatment and time had an interactive effect on regrowth (Supplemental Table 1), indicating the differences in regrowth between times were not the same for different preservation treatments. For instance, the regrowth of thalli preserved at -80 °C decreased to near zero by day seven, whereas thalli preserved at -20 °C regrew by day 30. In contrast, regrowth of thalli preserved at -4 °C had a high value even after 184 days ([Table 2](#)). The highest regrowth rate (23.09 ± 2.23%) was at -4 °C and it decreased to 3.59 ± 3.98% (15% DMSO) and 2.60 ± 3.10% (10% DMSO) at -20 °C. There were no significant differences between two-step and rapid cooling. The 15% DMSO concentration enhanced regrowth more noticeably at -20 °C ([Tables 2](#) and Supplemental Table 2).

Germlings were more vulnerable to freezing temperature than thalli. The PI was zero at -20 and -80 °C after one day of storage although the PI of germlings preserved at 4 °C remained high until day 30 (Supplemental Table 3). PI was also affected by storage duration (Friedman test, K = 25.159, df = 5, P < 0.001).

Regrowth of germlings preserved at -20 °C, -20 °C then -80 °C, and -80 °C was not measured due to the PI score of zero ([Table 3](#)). Germlings preserved at 4 °C had significant differences in regrowth between storage times (ANOVA, F = 50.698, df = 5, 48, P < 0.001); it had increased by 18.42% after day seven (Tukey HSD, P < 0.001) and had a further 10.46% rise by day 30 (Tukey HSD, P < 0.001). However, it decreased to 21.8 ± 3.0% for 92-day preservation (Tukey HSD, P < 0.001).

Preservation treatment and time interacted to affect gamete viability ([Fig. 1](#), Supplemental Tables 4 and 5). For instance, the viability of gametes preserved for one day at 4 °C, -20 °C with 5% DMSO, and -80 °C with 5% DMSO were 94.74 ± 4.30%, 56.14 ± 5.26%, and 12.48 ± 3.97%, respectively ([Fig. 1](#)). The highest viability (60.00 ± 23.32%) was at 4 °C.

4. Discussion

Successful preservation of seaweed thalli has been documented for relatively short-term storage duration ([Jo et al., 2003](#); [Kuwano et al., 1993, 1996](#)). Long-term preservation is poorly documented, although sporelings and apical segments of mature thalli of *Gracilaria foliiferae* were successfully stored in liquid nitrogen for four years ([Van der Meer and Simpson, 1984](#)). In the present study, cryopreservation with liquid nitrogen was not employed due to its failure in a preliminary experiment (data not shown). Instead, *Ulva rigida* was preserved at 4 °C and the freezing temperatures of -20 and -80 °C. Thallus survival decreased with time at -20 or -80 °C; all thalli were dead after seven

Table 1

Effects of preservation method on *Ulva rigida* pigmentation index where fully pigmented *Ulva* was given a score of 10 whereas completely white, dead fronds received a score of 0 (Van der Meer and Simpson, 1984). Samples were stored at the end temperature for times ranging from one to 184 days. Control is the reference measurement of the sample prior to storage. Data are the medians \pm interquartile range ($n = 9$).

Preservation method		Pigmentation index on each sampling day					
−80 °C	DMSO	1 d	2 d	7 d	30 d	92 d	184 d
	10%	2.0 \pm 1.0	2.0 \pm 1.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
−20 then −80 °C	15%	2.0 \pm 0.0	2.0 \pm 1.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
	10%	2.0 \pm 0.0	2.0 \pm 1.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
−20 °C	15%	2.0 \pm 0.0	2.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
	10%	8.0 \pm 1.0	8.0 \pm 1.0	7.0 \pm 1.0	0 \pm 0.0	0 \pm 0.0	0 \pm 0.0
4 °C	15%	9.0 \pm 0.0	9.0 \pm 1.0	8.0 \pm 1.0	0 \pm 0.0	0 \pm 0.0	0 \pm 0.0
	Control	10.0 \pm 0.0	10.0 \pm 0.0	10.0 \pm 0.0	10 \pm 0.0	10 \pm 0.0	9.0 \pm 1.0

days preservation at -80°C and 30 days preservation at -20°C . In addition, noticeable damage to thalli was found after only one day of preservation. A possible reason that this approach led to lower regrowth and shorter preservation periods than Van der Meer and Simpson's (1984) study is that different intermediate temperatures were used in the two-step cooling technique. Van der Meer and Simpson (1984) used -40°C whereas -20°C was used in the present study due to equipment limitation. Two-step cooling can substantially increase survival compared with rapid cooling as the low cooling rate from room temperature to an intermediate temperature allows sufficient time for intracellular water to externalise from the cell, reducing ice crystal formation (Taylor and Fletcher, 1999a). The intermediate temperature is usually set above -40°C as the cell membrane effectively becomes impermeable below this temperature (Karlsson et al., 1994). It appears that -40°C may be the preferable intermediate temperature as the highest survival of *Porphyra* thalli was found when samples were pre-frozen at -40°C before immersion in liquid nitrogen (Kuwano et al., 1996).

The regrowth rate of *Ulva* thalli or germlings was enhanced relative to the reference treatment after 4°C preservation for some time points (2–92 days for thalli and 7–30 days for germlings). This may potentially be related to gene regulation induced by cold shock, as it was found that overexpression of ERG10 enhanced survival and growth of yeast when frozen at -200°C for five days (Rodriguez-Vargas et al., 2002). Whereas the expression of heat shock proteins (HSP) was not determined in the current study, HSP gene clones are available for *U. pertusa* (Tominaga et al., 2010) and *U. prolifera* (Fu et al., 2011). The subsequent decrease in regrowth rate with time may relate to bacterial activity and the subsequent mortality of the *Ulva* germlings. Bacterial contamination usually occurs in low temperature preservation (Bhattarai et al., 2007).

Compared with other studies (Bhattarai et al., 2007; Taylor and Fletcher, 1999b), we preserved gametes at -20°C for a longer period. For instance, there was 100% mortality of *U. intestinalis* and *U. fasciata* spores after preservation at -20°C with 5% DMSO for three and five days respectively (Taylor and Fletcher, 1999b; Bhattarai et al., 2007). Despite the species differences and conceivable differences of freezing tolerance between gametes and spores, another possible reason is

down to the delayed germination due to freezing preservation. Germination of spores after culturing for three and seven days was employed in Taylor and Fletcher's (1999b) and Bhattarai et al.'s (2007) study respectively, to assess spore viability. We extended the culture period to 14 days, as gametes preserved at -20 or -80°C for more than two days did not germinate until they were cultured for 10–14 days. Therefore, a three- or seven-day culture might significantly underestimate spore viability. The viability of gametes stored at -80°C remained consistent, while it decreased with time at -20 and 4°C ; this could be attributed to bacteria remaining active at these temperatures (Bhattarai et al., 2007). Meanwhile, the continual loss of viability with time at -20 and 4°C might result from degradative enzymatic reactions in preserved cells. The rates of most chemical reactions decrease exponentially with decreasing temperature, including degradative enzymatic reactions. *Ulva* gametes frozen at -80°C seemed not to be affected by these reactions although Warren et al. (1997) proposed that cell deterioration cannot be completely prevented until -135°C .

Grout and Morris (1987) suggested that water loss is less effective during freezing of larger cells than smaller cells since larger cells have lower surface area to volume ratios. This makes larger cells more prone to intracellular ice formation during freezing, hence suffering more freezing injury. This might partially explain why gametes could be preserved for longer at -20 or -80°C compared with thalli or germlings. Vegetative cells are larger than spores and gametes since *Ulva* sporophytes usually produce 8 or 16 zoospores with 16 or 32 female gametes and 64 or 128 male gametes generated by cells of male and female gametophytes respectively (Smith, 1947). The size of vegetative cells and gametes in this study was $19.51 \pm 1.69 \mu\text{m} \times 14.05 \pm 1.81 \mu\text{m}$ (length \times width) and $5.93 \pm 0.74 \mu\text{m} \times 3.47 \pm 0.40 \mu\text{m}$ (length \times width) respectively; approximating to surface area to volume ratios of 0.387 for vegetative cells and 1.49 for gametes.

In addition, differences between gametes and adults might be due to lipid content and composition. Coalescence of lipid droplets has been considered to play a role in reducing the formation of intracellular ice in diatoms (McLellan, 1989) and thus it could theoretically result in an improvement in viability after cryopreservation. It was reported that

Table 2

Effects of preservation method on *Ulva rigida* thallus regrowth. Samples were stored at the end temperature for times ranging from one to 184 days and then cultured at 18°C , $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon light intensity, with a 16L: 8D photoperiod. Regrowth was calculated by recording the specific growth rate over a six-day culture period. Control represents the reference measurement of the sample prior to storage. Data are the means \pm SD ($n = 9$).

Preservation method		Regrowth (%) on each sampling day					
Temperature	DMSO	1 d	2 d	7 d	30 d	92 d	184 d
	10%	4.35 \pm 0.58	4.17 \pm 0.66	0.04 \pm 0.80	0.20 \pm 0.71	-0.22 \pm 0.49	-0.59 \pm 0.63
−20 then −80 °C	15%	4.45 \pm 0.66	4.26 \pm 0.63	0.04 \pm 0.84	0.15 \pm 0.75	-0.11 \pm 0.67	-0.38 \pm 0.58
	10%	4.40 \pm 0.53	4.21 \pm 0.67	0.04 \pm 0.80	0.20 \pm 0.71	-0.16 \pm 0.63	-0.27 \pm 0.49
−20 °C	15%	4.49 \pm 0.60	4.31 \pm 0.63	0.04 \pm 0.84	0.15 \pm 0.75	-0.11 \pm 0.67	-0.17 \pm 0.67
	10%	8.33 \pm 0.70	8.29 \pm 0.76	2.34 \pm 0.85	0.20 \pm 0.53	-0.06 \pm 0.56	-0.11 \pm 0.57
4 °C	15%	8.66 \pm 0.60	8.62 \pm 0.69	4.24 \pm 1.23	0.31 \pm 0.47	-0.11 \pm 0.57	-0.21 \pm 0.42
	Control	20.60 \pm 0.58	23.05 \pm 0.58	25.28 \pm 0.51	25.28 \pm 0.56	23.75 \pm 1.54	20.57 \pm 2.011

Table 3

Effects of preservation method on regrowth of *Ulva rigida* germlings. Samples were stored at the end temperature for times ranging from one to 184 days. Data are the means \pm SD ($n = 9$). Control represents the reference measurement of the sample prior to storage. – = Data not measured as all samples had died.

Preservation method		Regrowth (%) on each sampling day					
Temperature	DMSO	1d	2d	7d	30d	92d	184d
–80 °C	10%	–	–	–	–	–	–
	15%	–	–	–	–	–	–
–20 then –80 °C	10%	–	–	–	–	–	–
	15%	–	–	–	–	–	–
–20 °C	10%	–	–	–	–	–	–
	15%	–	–	–	–	–	–
4 °C		27.91	28.17	33.36	36.85	21.83	20.52
		± 2.88	± 2.57	± 1.93	± 2.03	± 3.03	± 3.32
Control		27.27 ± 2.40					

lipid droplets avoided or decreased modification of cell morphology and thus enhanced freezing tolerance of *Undaria pinnatifida* gametophytes (Ginsburger-Vogel et al., 1992). After release, gametes and spores need to locate and adhere to a surface. Most *Laminaria* zoospores have only one or two chloroplasts which leads to a very low photosynthetic capacity that cannot supply the whole organism with energy for movement, settlement, and germination. Therefore, there should be endogenous energy reserves to support these processes. It has been found that zoospores of the palm kelp *Pterygophora californica* consumed 43% of neutral lipid to fuel swimming over 30 h in the dark (Reed et al., 1999). Compared to vegetative cells, there would be a higher lipid content to fuel these activities. This hypothesis was verified by Steinhoff et al. (2011) who demonstrated a decrease in lipid during development from spores to gametophytes in *Saccharina latissima*. Furthermore, freezing tolerance could be related to lipid composition as it will affect the permeability of the plasma membrane to water. For instance, *Saccharomyces cerevisiae* enriched in ergosterol or stigmasterol rather than campesterol or cholesterol had higher viability (Calcott and Rose, 1982). Different fatty acid profiles between *S. latissima* zoospores and gametophytes were reported, with a decrease in the fatty acid 18:1(n-9) from 45 to 30% (Steinhoff et al., 2011). This decrease in unsaturated fatty acids might lead to a decline in the ability of gametophytes to endure cold temperatures compared to zoospores.

On the other hand, *Ulva* thalli or germlings performed better than gametes when preserved at 4 °C. This difference may be attributed to bacterial activity as bacteria were present in all three preservation methods. Bhattacharai et al. (2007) reported that the addition of 100 µg ml⁻¹ ampicillin enhanced the viability of *U. fasciata* and *U. pertusa* spores stored at 4 °C. When preserving the thalli or germlings, the bacterial biofilms formed in the flasks were removed during monthly media renewal, avoiding or decreasing harm from bacteria. Nutrient

limitation might be another possible reason for the decrease in gamete preservation with time. Although low temperature and light level inhibited photosynthesis, metabolic activities continued at a low rate. The cells would keep consuming nutrient in the media – that was not renewed for gamete preservation.

The higher DMSO concentration (15%) promoted thalli regrowth over the 10% concentration. The thalli survival of *Gracilaria tikvahiae* (Van der Meer and Simpson, 1984) and *Porphyra yezoensis* (Kuwano et al., 1993) also increased with DMSO concentration but the optimal level was around 10% for both species. On the other hand, 10% DMSO reduced *Ulva* gamete viability compared to 5%. Likewise, Taylor and Fletcher (1999b) found that 5% DMSO afforded greater protection than 10% for *U. intestinalis* zoospores.

The present study has, for the first time, investigated the feasibility of different preservation methods for *U. rigida* thalli, germlings, and gametes. Thalli, germlings, and gametes can be preserved at 4 °C for an extended period (184 days) whereas only gametes could be stored at –80 °C for 184 days. Germling and gamete preservation occupies less space with quicker regrowth, but requires more time for the plant to reach a harvestable size. Low temperature (4 °C) preservation increased thalli and germling viability and was well suited to long-term preservation. This low cost, low-tech technique (defined as not requiring access to liquid nitrogen) is more accessible to seaweed farmers. In addition, the pigment index was confirmed as a simple but effective method to determine seaweed survival.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.aquaculture.2017.02.012>.

References

- Arbault, S., Renard, P., Perez, R., Kass, R., 1990. Cryopreservation trials on the gametophytes of the food alga *Undaria pinnatifida* (Laminariales). Aquat. Living Resour. 3, 207–215.
- Bhattacharai, H.D., Paudel, B., Hong, Y.-K., Shin, H.W., 2007. A simple method to preserve algal spores of *Ulva* spp. in cold storage with ampicillin. Hydrobiologia 592, 399–404.
- Calcott, P.H., Rose, A.H., 1982. Freeze-thaw and cold-shock resistance of *Saccharomyces cerevisiae* as affected by plasma membrane lipid composition. J. Gen. Microbiol. 128, 549–555.
- Day, J.G., 1998. Cryo-conservation of microalgae and cyanobacteria. Cryolett. Suppl. 1, 7–14.
- Fu, W., Shuai, L., Yao, J., Zheng, B., Zhong, M., Duan, D., 2011. Molecular cloning and expression analysis of a cytosolic Hsp70 gene from *Ulva pertusa* (Ulvophyceae, Chlorophyta). J. Appl. Phycol. 23, 681–690.
- Ginsburger-Vogel, T., Arbault, S., Perez, R., 1992. Ultrastructural study of the effect of freezing/thawing on the gametophyte of the brown alga *Undaria pinnatifida*. Aquaculture 106, 171–181.
- Grout, B.W.W., Morris, G.J., 1987. Freezing and cellular organization. In: Grout, B.W.W., Morris, G.J. (Eds.), *The Effects of Low Temperatures on Biological Systems*. Edward Arnold, London.
- Hiraoka, M., Enomoto, S., 1998. The induction of reproductive cell formation of *Ulva pertusa* Kjellman (Ulvales, Ulvophyceae). Phycol. Res. 46, 199–203.
- Jo, Y.-H., Kang, S.-P., Seo, T.-H., Choi, S.-J., Kho, K.H., Kuwano, K., Saga, N., Kim, M.-Y., Shin, J.-A., 2003. Cryopreservation of sporothallii of the genus *Porphyra* (Bangiales, Rhodophyta) from Korea. Algae 18, 321–332.
- Karlsson, J.O.M., Cravalho, E.G., Toner, M., 1994. A model of diffusion-limited ice growth inside biological cells during freezing. J. Appl. Phys. 75, 4442–4455.
- Kuwano, K., Aruga, Y., Saga, N., 1992. Preliminary study on cryopreservation of the conchocelis of *Porphyra yezoensis*. Bull. Jpn. Soc. Sci. Fish. 58, 1793–1798 (Japan).
- Kuwano, K., Aruga, Y., Saga, N., 1993. Cryopreservation of the conchocelis of the marine alga *Porphyra yezoensis* Ueda (Rhodophyta) in liquid nitrogen. Plant Sci. 94, 215–225.
- Kuwano, K., Aruga, Y., Saga, N., 1994. Cryopreservation of the conchocelis of *Porphyra* (Rhodophyta) by applying a simple prefreezing system. J. Phycol. 30, 566–570.

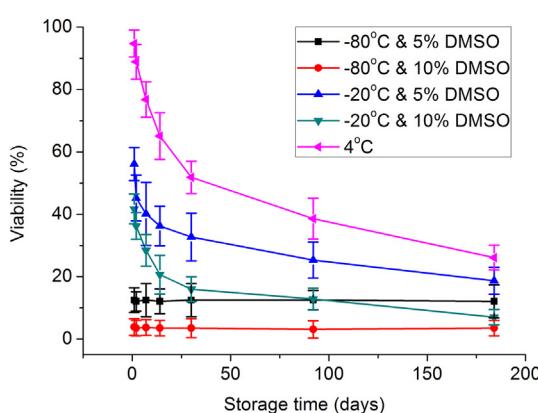


Fig. 1. Effects of different preservation methods on the viability of *Ulva rigida* gametes. Samples were stored at the end temperature for times ranging from one to 184 days. The error bars indicate the standard deviations ($n = 9$).

- Kuwano, K., Aruga, Y., Saga, N., 1996. Cryopreservation of clonal gametophytic thalli of *Porphyra* (*Rhodophyta*). *Plant Sci.* 116, 117–124.
- Lee, Y.N., Nam, K.W., 2016. Cryopreservation of gametophytic thalli of *Ulva prolifera* (*Ulvales, Chlorophyta*) from Korea. *J. Appl. Phycol.* 28, 1207–1213.
- McLellan, M.R., 1989. Cryopreservation of diatoms. *Diatomol. Res.* 4, 301–318.
- Migita, S., 1964. Freeze-preservation of *Porphyra* thalli in a viable state. I. Viability of *Porphyra tenera* preserved at low temperature after freezing in seawater and freezing under half-dried conditions. *Bull. Fac. Fish. Hokkaido Univ.* 17, 44–54.
- Migita, S., 1966. Freeze-preservation of *Porphyra* thalli in viable state-II. Effect of cooling velocity and water content of thalli on the frost resistance. *Bull. Fac. Fish. Hokkaido Univ.* 21, 131–138.
- Migita, S., 1967. Viability and spore liberation of the conchocelisphase of *Porphyra tenera* freeze preserved in seawater. *Bull. Fac. Fish. Hokkaido Univ.* 22, 33–43.
- Reed, D.C., Brzezinski, M.A., Coury, D.A., Graham, W.M., Petty, R.L., 1999. Neutral lipids in macroalgal spores and their role in swimming. *Mar. Biol.* 133, 737–744.
- Renard, P., Arbault, S., Kaas, R., Perez, R., 1992. Une méthode pour la cryoconservation des gamétophytes de l'algue alimentaire *Undaria pinnatifida* (Laminariales). *Comptes rendus de l'Académie des sciences. Série 3. Sciences de la vie.* 315, 445–451.
- Rodriguez-Vargas, S., Estruch, F., Randez-Gil, F., 2002. Gene expression analysis of cold and freeze stress in Baker's yeast. *Appl. Environ. Microbiol.* 68, 3024–3030.
- Smith, G.M., 1947. On the reproduction of some Pacific coast species of *Ulva*. *Am. J. Bot.* 34, 80–87.
- Steinhoff, F.S., Graeve, M., Wiencke, C., Wulff, A., Bischof, K., 2011. Lipid content and fatty acid consumption in zoospores/developing gametophytes of *Saccharina latissima* (Laminariales, Phaeophyceae) as potential precursors for secondary metabolites as phlorotannins. *Polar Biol.* 34, 1011–1018.
- Taylor, R., Fletcher, R.L., 1999a. Cryopreservation of eukaryotic algae—a review of methodologies. *J. Appl. Phycol.* 10, 481–501.
- Taylor, R., Fletcher, R.L., 1999b. A simple method for the freeze-preservation of zoospores of the green macroalgae *Enteromorpha intestinalis*. *J. Appl. Phycol.* 11, 257–262.
- Tominaga, H., Coury, D.A., Amano, H., Kakimoto, M., 2010. Isolation and characterization of a cDNA encoding a heat shock protein 70 from a sterile mutant of *Ulva pertusa* (*Ulvales, Chlorophyta*). *Ecotoxicol.* 19, 577–588.
- Van der Meer, J.P., Simpson, F.J., 1984. Cryopreservation of *Gracilaria tikvahiae* (*Rhodophyta*) and other macrophytic marine algae. *Phycologia* 23, 195–202.
- Vigneron, T., Arbault, S., Kaas, R., 1997. Cryopreservation of gametophytes of *Laminaria digitata* (L.) Lamouroux by encapsulation dehydration. *CryoLetters* 18, 93–98.
- Wang, B., Zhang, E., Gu, Y., Ning, S., Wang, Q., Zhou, J., 2011. Cryopreservation of brown algae gametophytes of *Undaria pinnatifida* by encapsulation–vitrification. *Aquaculture* 317, 89–93.
- Warren, A., Day, J.C., Brown, S., 1997. Cultivation of protozoa and algae. In: Hurst, C.J., K. G.H., McInerney, M.J., Stetzenbach, L.D., Walter, M.V. (Eds.), *Manual of Environmental Microbiology*. ASM Press, Washington, DC, pp. 61–71.
- Zhuang, Y., Gong, X., Zhang, W., Gao, W., 2015. Cryopreservation of filaments of *Scytoniphon lomentaria* by vitrification. *J. Appl. Phycol.* 27, 1337–1342.