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1 **Germplasm Cryopreservation of Macroalgae for Aquaculture Breeding and Natural**
2 **Resource Conservation: A Review**
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18 **Running title**: Review of Macroalgal Germplasm Cryopreservation
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20 **ABSTRACT**

21

22 The expansion of the global macroalgal aquaculture and climate change creates the need for
23 germplasm preservation of valuable aquaculture strains and maintenance of natural biodiversity.
24 Compared to the large number of studies in fish and shellfish species, relative few studies have
25 been conducted on the macroalgal germplasm cryopreservation. The first cryopreservation of
26 macroalgae to -75 °C was reported on *Neopyropia tenera* (formerly called *Porphyra tenera*) in
27 1964. To date, a total of 34 studies reported germplasm cryopreservation in 33 species, including
28 Chlorophyta (7 species), Ochrophyta (14 species), and Rhodophyta (12 species). The goal of this
29 review was to summarize the published studies on macroalgal germplasm cryopreservation,
30 compare the reported protocols for the cryopreservation process, and identify the factors
31 affecting post-thaw viability. Overall, the macroalgal germplasm for cryopreservation included
32 haploid or diploid thalli, spores, and gametes. Cryotubes (1.5-ml or 2-ml) have been widely used
33 to package germplasm samples for cooling and storage in most studies, and the 0.5-ml straws
34 and 5-ml cryotubes have been used in several studies. Two approaches (programmable controlled
35 cooling and vitrification) were employed for macroalgal germplasm cryopreservation. A two-step
36 programmable controlled cooling (e.g., from initial culture temperature to a frozen temperature,
37 such as -40 °C, and then directly plunging into liquid nitrogen at -196 °C) was determined to be
38 an effective cooling strategy. Vitrification, a super rapid cooling for a sample to form
39 non-crystalline amorphous solid, was applied on macroalgal germplasm cryopreservation with
40 sample encapsulation and dehydration. Survival of post-thaw samples varied significantly in
41 different studies. Based on research updates, recommendations are made for future research. It is
42 expected that this review can serve as a foundation for future germplasm banking of macroalgae
43 for aquaculture and biodiversity preservation.

44 **Key words**: Macroalgae, Seaweed, Germplasm, Aquaculture, Cryopreservation.

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71 **1. Introduction**

72

73 Macroalgae, commonly known as seaweeds, are multicellular marine green, red, and brown
74 algae consisting of complex life cycles, which include multicellular or siphonous macrothalli
75 (Hurd et al., 2014). They vary in size from a few millimeters to ~60 meters (e.g., *Macrocystis*
76 *pyrifera*) (Schiel and Foster, 2015). Based on the pigments in the chloroplast, macroalgae are
77 classified into three groups: Chlorophyta (green algae), Ochrophyta (brown algae), and
78 Rhodophyta (red algae) (Baweja et al., 2016; Graham et al., 2019). Macroalgae play a significant
79 role in the ecosystem as ecological engineers (Umanzor et al., 2019), primary producers
80 (Rosenzweig et al., 2008), habitat and structure providers (Dayton et al., 1984), nutrient cyclers
81 (Paine, 1969), ecosystem services (Neori et al., 2004; Kim et al., 2014; 2015; Kim et al., 2017;
82 Park et al., 2018; Kim et al., 2019; Park et al., 2021; Racine et al., 2021); essential connectors in
83 the food chain for invertebrates and pelagic organisms, and shoreline buffers from storms
84 (Steneck et al., 2002; Smale et al., 2013). Furthermore, some macroalgae also have great
85 economic value as direct food sources, being used as polysaccharide additives, or food
86 ingredients for human consumption because of their nutritional value, richness in proteins,
87 vitamins, minerals, and other organic substances (MacArtain et al., 2007; Hafting et al., 2015;
88 Wells et al., 2017; Naylor et al., 2021). Additionally, macroalgae have been used in the
89 industries as fertilizers (Pereira and Yarish, 2008; Kim et al., 2017; Buschmann and Camus,
90 2019), polysaccharides (Jönsson et al., 2020), oligosaccharides (Jiao et al., 2011), algal
91 hydrocolloids (Roesijadi et al., 2010), minerals (Circuncisão et al., 2018), pharmaceuticals
92 (McHugh, 2003), medical therapeutics (Vera et al., 2011), animal feeds (Vijn et al., 2020), and
93 textile industries (Bixler and Porse, 2011). Overall, macroalgae are valuable and promising
94 natural resources in diverse fields (Leandro et al., 2020).

95 To date, over 200 species of macroalgae have been harvested as food or for industrial uses
96 (Sahoo et al., 2002; Ferdouse et al., 2018). Worldwide, macroalgal production in 2018 was 32.4
97 million tonnes (FAO, 2020) including the Japanese kelp *Saccharina japonica* (35.3% by
98 production), *Eucheumoid* seaweeds (29.1%; *Kappaphycus alvarezii*, *Eucheuma denticulatum* and
99 other *Eucheumoid* spp.), *Gracilaria* spp. (10.7%), nori *Neopyropia tenera* and *Neopyropia*
100 *yezoensis* (formerly called *Porphyra tenera* and *Porphyra yezoensis, respectively*) (8.9%),
101 *Sargassum fusiforme* (0.8%), and other algal species (Buschmann et al., 2017; Kim et al., 2017;

102 Critchley et al., 2019; FAO, 2020). Macroalgal aquaculture is practiced in Asian countries
103 (principally China, Indonesia, Korea, Philippines, Japan, and Malaysia), with total production
104 being tripled from 2000 (10.6 million tonnes) to 2018 (32.4 million tonnes) (FAO, 2020). In
105 recent years, seaweed aquaculture has been growing rapidly in European and North American
106 countries for food, feed, bioenergy, nutrient bioextraction, and industrial uses (Grebe et al., 2019;
107 Piconi et al., 2020; Vijn et al., 2020). With the steady growth of macroalgal aquaculture,
108 breeding programs are being developed with different genetic manipulations to produce strains
109 or lines suitable in different environments for improved productivity and quality (Mao et al.,
110 2020). Germplasm from these aquaculture lines need to be preserved to promote sustainable
111 seaweed aquaculture (Wade et al. 2020).

112 As many other marine species, macroalgae are also facing biodiversity losses at alarming
113 rates (De Paula et al., 2020) due to multiple stressors, such as warming sea surface temperatures,
114 pollutants, overharvesting, and other anthropogenic disturbances (Smale et al., 2013; Krumhansl
115 et al., 2016; Wade et al., 2020). Potential consequences include changes of ecological structure,
116 loss of genetic diversity, ecological function and services provided by macroalgae, and
117 eventually extinction (Díez et al., 2012; Assis et al., 2017; Steneck et al., 2019). Loss of genetic
118 diversity in macroalgae was identified in several farmed seaweed species due to limited space in
119 germplasm banks or continuous inbreeding (Cardinale, 2011; Valero et al., 2017).

120 The need for germplasm banking of macroalgae has been emphasized for the preservation of
121 cultivars, biodiversity conservation and ecosystem restoration, and diverse research applications
122 (Wade et al., 2020). Preservation of cultivated strains from the aquaculture industry is an
123 important strategy to maintain this economically valuable germplasm in perpetuity (Wade et al.,
124 2020) and serve as the repositories of genetic variation (Tanksley and McCouch, 1997).
125 Preservation of wild types for genetic diversity can provide biological insurance against
126 environmental stresses, natural weather disasters, and unpredictable accidents from maritime
127 industries (Barrento et al., 2016). Cryopreservation is a technology referring to the preservation
128 of biological materials, including germplasm, at extremely low temperatures (commonly in
129 liquid nitrogen at –196 °C). Application of cryopreservation technology for germplasm has
130 played a significant role in human infertility treatment, maintenance of biological diversity,
131 preservation of genetic resources, assistance of breeding programs, and conservation of
132 imperiled species (Yang and Tiersch, 2020). For macroalgae, germplasm cryopreservation is

133 becoming an important and acceptable tool for long-term germplasm banking and conservation
134 (O'Connell et al., 2020).
135 In this review, the development of germplasm cryopreservation in macroalgae is evaluated
136 and summarized for macroalgae aquaculture production and natural resource conservation.
137 Literature searches were performed in databases from Web of Science Core Collection and
138 Google Scholar with keywords of "macroalgae (or macroalgal), vitrification, cryopreservation
139 (or cryopreserve), Chlorophyta, Ochrophyta, and Rhodophyta". The findings and results in
140 published studies on macroalgal germplasm cryopreservation will be summarized and compared
141 at each step of the cryopreservation process (Tables 3, 4, and 5 for Chlorophyta, Ochrophyta, and
142 Rhodophyta). The factors affecting post-thaw viability and repeatability were evaluated, and
143 directions for future research will be discussed. It is anticipated that this review can serve as a
144 foundation for future germplasm banking of macroalgae for aquaculture and natural resource
145 conservation. For microalgal cryopreservation, the interested readers are referred to other related
146 review references or book chapters (Day et al., 1999; Day, 2004; 2007; Day et al., 2010;
147 Fernandes et al., 2019).
148
149 **2. Development of germplasm cryopreservation in macroalgae**
150
151 ***2.1. Approach for germplasm cryopreservation in macroalgae***
152
153 The history and principles of cryopreservation technology have been introduced and reviewed
154 in other publications (Pegg, 2002; Yang and Tiersch, 2020). During the cooling process, two
155 possible factors are responsible for cell injury: (1) solute effect, which injures cells by high solute
156 concentrates when cells are cooled at a controlled cooling rate; and (2) intracellular ice formation,
157 which injures cells via intracellular ice crystals when cells are cooled at fast cooling rates (Pegg,
158 2002). Based on experimental observations, a two-factor hypothesis was proposed and was
159 illustrated with follow-up experiments on cryopreservation for different types of cells at a wide
160 range of cooling rates (Leibo, 1976). Since then, this hypothesis has been recognized as the basis
161 of cryopreservation, and two cooling approaches have been developed for germplasm
162 cryopreservation (Pegg, 2002).
163 1) *Cryopreservation by controlled cooling-rates.*

164 This approach was based on the two-factor hypothesis. The optimized cooling rates can be
165 experimentally determined (Yang et al., 2012) or theoretically predicted (Thirumala et al., 2005) to
166 increase post-thaw cell survival (Mazur, 2004). For macroalgae, most of the cryopreservation
167 studies used a two-step cooling rate method. The detailed summary will be illustrated in Section 3.
168 2) *Vitrification by cooling at ultra-rapid rates.*
169 This approach involves intracellular formation of a stable *glass* state (i.e., the solidification of a
170 liquid in the absence of crystallization) (Rall and Fahy, 1985) and used for mammalian
171 cryopreservation of oocytes (Kuwayama et al., 2005), embryos (Kasai and Mukaida, 2004), and
172 blastocyst (Dal Canto et al., 2019). Vitrification can be achieved through osmotic dehydration by
173 using penetrating cryoprotectants or cooling at ultrafast rates, and complemented with ultra-rapid
174 warming (Mazur and Paredes, 2016). For macroalgae, vitrification was conducted by
175 encapsulating samples into 3% calcium alginate beads, which are cooled in liquid nitrogen directly
176 after dehydration (Wang et al., 2000; Zhang et al., 2008). The detailed summary will be illustrated
177 in Section 3.
178
179 ***2.2. Germplasm materials for cryopreservation in macroalgae***
180
181 Macroalgae come in many varieties in sizes and structures. The smallest macroalgae are only
182 a few millimeters with a simple filamentous structure, while the largest macroalgae (e.g., giant
183 kelp measures up to 60 meters) could have complex structures with specific tissues (Mouritsen,
184 2013) and cells containing several nuclei and organelles (Baweja et al., 2016).
185 Generally, macroalgae have varying life history strategies which include gametic meiosis,
186 sporic meiosis, or zygotic meiosis. Life histories of macroalgae that have sporic meiosis may
187 have haploid and diploid generations that are isomorphic or heteromorphic life cycles (See
188 detailed figures in Redmond et al., 2014; Graham et al., 2019). The sporophyte produces haploid
189 meiospores, which upon germination grow directly into dioecious gametophytes. Gametophytes
190 produce mitotically a range of gamete types depending upon the species (isogametes,
191 anisogametes or oogametes). After gametes fuse, a diploid zygote grows mitotically into diploid
192 sporophytes. If the haploid gametophyte is morphologically similar to the diploid sporophyte,
193 such organisms are characterized by an alternation of isomorphic generations. If the haploid and
194 diploid stages are dissimilar, the organism exhibits an alternation of heteromorphic generations.

195 In addition, algae characterized by an alternation of generations can reproduce asexually via
196 mitosis - or gametophytes can produce haploid parthenogametes. Based on the life cycles,
197 reproduction features, and capability for regenerations in macroalgae, germplasm materials for
198 cryopreservation could be any stage of the algal thallus (sporophyte or gametophyte thalli,
199 meiotic spores, mitotic spores, and/ or parthenogametes).
200
201 ***2.3. History of macroalgal germplasm cryopreservation***
202
203 Seed banking in terrestrial plants has been emphasized for several decades (Tanksley and
204 McCouch, 1997). For algae, germplasm cryopreservation has been studied in many microalgae
205 species, and germplasm repositories have been established in several species of economic
206 importance (Barrento et al., 2016). Comparatively, fewer germplasm cryopreservation studies
207 have been reported on macroalgae with limited success for long-term repository establishment
208 (Taylor and Fletcher, 1999b; Day and Harding, 2008).
209 Macroalgal cryopreservation was firstly reported in 1960 on the survival of green alga *Ulva*
210 *pertusa* after exposure to low temperatures at -5, -10, -15, -20, and -28 °C by direct cooling
211 without cryoprotectants (Terumoto, 1960). The results indicated that *Ulva pertusa* could tolerate
212 low temperature to -10 °C for at least 24 h without cell injury. Later on, *Ulva intestinalis*
213 (formerly called *Enteromorpha intestinalis*), was found to tolerate -20 °C for 24 h without cell
214 injury, and 50% of cells survived after 3 days freezing at -20 °C (Terumoto, 1961), indicating the
215 different resistance to low temperatures between *U. pertusa* and *U. intestinalis*. Therefore, direct
216 comparisons of three green algae and five red algae in their resistance were conducted and
217 significant differences were found (Terumoto, 1964). Since no efforts were made for long-term
218 cryopreservation with cryoprotectants, those studies were categorized as frost resistance or cold
219 storage (Table 1).
220 The first cryopreservation of macroalgae was reported in *Neopyropia tenera* (formerly
221 referred to as *Porphyra tenera*) with glycerol or glucose at 2.5, 5, 10, and 20% as cryoprotectants
222 (Migita, 1964). The findings were: 1) vegetative and rhizoidal cells of thalli showed higher
223 resistance to freezing temperatures than carposporangial cells. Neutral spores showed higher
224 resistance than carpospores and *conchocelis* filaments; 2) cells cryopreserved to -20 °C showed
225 higher post-thaw survival than that to -75 °C; 3) half-dried cells remained fully viable after

226 cryopreservation at -20 °C for 4 months; and 4) glucose, but not glycerol, showed protection of
227 cells against freezing (Migita, 1964). Following this study, effects of cooling rate and water
228 content in thalli were studied in *Neopyropia tenera* (= *Pyropia tenera*), *Neopyropia yezoensis*
229 (=*Pyropia yezoensis*), and *Phycocalidia suborbiculata* (=*Pyropia suborbiculata*) (Migita, 1966).
230 Additionally, sucrose, glycerol, NaCl, and ethylene glycol were used to cryopreserve *Neopyropia*
231 *yezoensis*, NaCl and ethylene glycol did not cause cell injury, but sucrose, glycerol, and distilled
232 water resulted in a fatal frost-injury to the cells (Terumoto, 1965). Since the early 1980s, a
233 two-step cooling approach (van der Meer and Simpson, 1984) has been largely employed in
234 many publications to cool macroalgae at cryogenic temperatures (-196 °C) with varied post-thaw
235 survival. In recent studies, different pre-treatments before cooling, cryoprotectants, and cooling
236 approaches were explored.

237 Through an intensive literature search, a total of 35 papers have been found on macroalgal
238 cryopreservation (Table 2). These 35 publications addressed germplasm cryopreservation in a
239 total of 33 species, including 7 green algae (6 publications, Table 3), 14 brown algal species (15
240 publications, Table 4), and 12 red algae (13 publications, Table 5). Because of their economic
241 value as marine aquaculture crops, *Neopyropia yezoensis*, *Undaria pinnatifida*, and *Saccharina*
242 *japonica* were the most studied species for germplasm cryopreservation (Table 2). Besides these
243 research publications, one review paper (Taylor and Fletcher, 1999b), three book chapters or
244 conference proceedings (Kuwano and Saga, 2000; Day, 2018; Paredes et al., 2021), and one
245 perspective paper (Wade et al., 2020) were found on macroalgal germplasm cryopreservation.

246 Despite these publications on macroalgal germplasm cryopreservation, significant variations
247 in post-thaw survival were reported and there have been no uniformed standardized protocols
248 even for the same species. Therefore, long-term germplasm repositories in macroalgae are still
249 lacking (Wade et al., 2020). Currently, macroalgal collections in many institutes are held as live
250 collections with a few species cryopreserved, such as the Culture Collection of Algae and
251 Protozoa (ccap.ca.uk), the Roscoff Culture Collection (http://roscoff-culture-collection.org/), and
252 the Bigelow Laboratory for Ocean Sciences (https://www.bigelow.org/). Maintenance of live
253 macroalgae needs space, manpower and is prohibitively expensive. Furthermore, there is always
254 a risk of losing certain strains or species due to catastrophic failure of the environmental systems
255 or contamination by human error. Therefore, further studies on standardization and

256 quantification of cryopreservation procedures are needed to increase the protocol repeatability
257 for successful application on germplasm banking.
258
259 **3. Germplasm cryopreservation of macroalgae**
260
261 Cryopreservation procedures involve a series of steps that are connected to each other and
262 need to be optimized by experimental trials for the species of interest. These steps include: 1)
263 germplasm sample collection; 2) selection of cryoprotectants by evaluating the acute toxicity and
264 cryoprotective function during cooling process; 3) packaging of germplasm samples after mixing
265 with cryoprotectants; 4) cooling process at suitable cooling rates by evaluating post-thaw
266 survival; 5) thawing of frozen samples at different temperatures; and 6) viability assays or
267 post-thaw amendments of samples before further use. Optimization of each cryopreservation step
268 is crucial for protocol development with high post-thaw viability because these steps are
269 interconnected and any error at any step could result in final failure (Leibo and Pool, 2011). In
270 general, the optimized conditions at each step varied among different species, cell types (cell size,
271 cell wall and cytoplasm membrane, etc.), and even the same samples when handling differently.
272 The summary of macroalgal cryopreservation at each step was reviewed as follows.
273
274 ***3.1. Germplasm collection and preparation for cryopreservation***
275
276 The targeted macroalgal germplasm for cryopreservation needs to be specific to their life
277 cycle and reproduction characteristics (Wade et al., 2020). See section 2.2 the statement of life
278 cycle and potential germplasm materials for cryopreservation.
279
280 *3.1.1. Diploid or haploid thalli*
281 Haploid and diploid thalli are present at different stages of the life cycle for macroalgae. For
282 Chlorophyta and Ochrophyta macroalgae (Tables 3 and 4), the germplasm for cryopreservation
283 included haploid gametophytic thalli (Wang et al., 2005; Zhang et al., 2007b; Zhang et al., 2007a;
284 Zhang et al., 2008; Nanba et al., 2009; Barrento et al., 2016; Lee and Nam, 2016; Gao et al.,
285 2017; Visch et al., 2019), and haploid parthenosporophyte thalli in *Ectocarpus* species (Heesch
286 et al., 2012). For Rhodophyta, including *Neopyropia* and *Porphyra* spp. (Table 5), diploid

287 conchocelis was the most common germplasm for cryopreservation (Kuwano et al., 1993; Wang
288 et al., 2000; Zhou et al., 2007; Choi et al., 2013), and gametophytic thalli and free-living diploid
289 conchocelis filaments were cryopreserved with relatively higher post-thaw viability (Kuwano et
290 al., 1996; Jo et al., 2003) (Table 5). For other Rhodophyta macroalgal species, such as *Gracilaria*
291 *corticata*, *Gracilaria tikvahiae* and *Hypnea musciformis*, apical segments of mature thalli were
292 used for cryopreservation (van der Meer and Simpson, 1984; Lalrinsanga et al., 2009).
293 Preparation of diploid or haploid thalli were performed by cleaning with sterilized seawater
294 and cutting into 1-2 mm fragments using blenders or single edge razor blades. In some reports,
295 the fragments after cutting were cultured in sterilized medium with or without antibiotics for a
296 period of days or months before being used for cryopreservation.
297
298 *3.1.2. Spores, gametes, and zygotes,*
299 For most animals, germplasm such as gametes, embryos, and larvae are the primary focus
300 for cryopreservation. For macroalgae, spores, gametes, and zygotes were suitable germplasm for
301 long-term cryopreservation. To date, several studies reported successful cryopreservation or cold
302 storage of meiospores from *Saccharina japonica* (Zhang et al., 2007b), *Ulva intestinalis* (Taylor
303 and Fletcher, 1999a), *Ulva fasciata* and *Ulva pertusa* (Bhattarai et al., 2007), gametes from *Ulva*
304 *rigida* (Gao et al., 2017) and zygotes (embryos) from *Fucus edentatus* (Bird and McLachlan,
305 1974). In addition, meiospores of kelps were reported cryopreserved successfully in the Bigelow
306 Laboratory (www.bigelow.org, personal communication with M. Lomas).
307 For Chlorophyta (Table 3), zoospores of *Ulva fasciata* and *Ulva pertusa* cooled down to
308 4 °C in f/2 seawater medium with ampicillin (100 μg mL^{-1}) showed a viability of 42-61% after
309 storage for 100 days (Bhattarai et al., 2007). Zoospores of *Ulva intestinalis* cooled to -20 °C
310 showed a viability of over 40% after storage for 5 weeks (Taylor and Fletcher, 1999a), and
311 gametes of *Ulva rigida* cooled to -20 °C and -80 °C showed 7.0 - 18.7% and 3.5–12.1%
312 post-thaw viability (Gao et al., 2017). Released gametes and zoospores in *Ulva* species were
313 collected by concentrated them using a point light source (Hiraoka and Enomoto, 1998). For
314 Ochrophyta (Table 4), meiospores of *Saccharina japonica* cryopreserved in liquid nitrogen by
315 gradual cooling at 0.25-5.0 °C min^{-1} to -60 °C followed by plunging directly into liquid nitrogen
316 showed a 13-50 % viability after 24 h (Zhang et al., 2007b). For *Saccharina japonica*,
317 meiospores were released from 10-cm^2 pieces of sorus tissue in sterilized seawater at 8 °C and

318 were collected by filtering through a 30-µm cell strainer (Zhang et al., 2007b). For Rhodophyta
319 macroalgae (Table 5), there has been no study on spores or gametes cryopreservation.
320
321 ***3.2. Choice of cryoprotectants and evaluation of acute toxicity***
322
323 The use of cryoprotectants is essential for cryopreservation technology since the first
324 application of glycerol (20%) as cryoprotectant for fowl semen cryopreservation (Polge et al.,
325 1949). Since then, cryopreservation has been investigated in many hundreds of different species,
326 and the use of cryoprotectant is almost universal for cryopreservation technology. For different
327 cell types and species for cryopreservation, effective cryoprotectant types and concentrations
328 need to be determined through systematic experimentation based on cryoprotectant toxicity,
329 molecular weight, and permeability (See reviews in Pegg, 2002; Elliott et al., 2017; Yang and
330 Tiersch, 2020). Depending on cryoprotectant toxicity, cell sensitivity, and osmotic pressures,
331 methods to mix cryoprotectants and biological cell suspension could be conducted in different
332 ways at different temperatures. After mixing with cryoprotectants, sample suspensions usually
333 require a specific time, which is called "equilibration time", to allow cryoprotectants and
334 samples to interact with one another before cooling. Systematic assessment of the acute toxicity
335 of cryoprotectants on fresh samples is an effective way to screen cryoprotectant types,
336 concentrations, and equilibration time for germplasm cryopreservation.
337 For macroalgae, choice of cryoprotectants in most studies were based on peers' results with
338 DMSO, glycerol and sugars. Acute toxicity evaluation for screening cryoprotectants were
339 reported in only two studies (Zhang et al., 2007b; Choi et al., 2013)). For *Neopyropia yezoensis*,
340 a total of 10 cryoprotectants were evaluated on gametophytic thalli, and a combination of 5%
341 DMSO and 20% diglycerol showed the least toxicity with a 94.6% post-exposure viability (Choi
342 et al., 2013). For *Saccharina japonica*, five cryoprotectants (DMSO, glycerol, sucrose, dextrose,
343 and sorbitol) were showed toxicity on meiospores, and DMSO showed the lowest level of
344 toxicity. After exposure to 5% DMSO for 50 min or 10% DMSO for 15 min, about 70% of the
345 meiospores developed into gametophytes (Zhang et al., 2007b).
346
347 *3.2.1. Types and concentrations of cryoprotectant*

348 The cryoprotectant types used for macroalgae germplasm cryopreservation include DMSO,
349 glycerol, methanol, ethylene glycol, polyethylene glycol, propylene glycol, dipropylene glycol,
350 3-methyl-1,3-butanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, and 2,3-butanediol
351 (see summaries in Tables 3, 4, and 5) with DMSO reported in majority of the publications
352 (Taylor and Fletcher, 1999a). Additionally, amino acids (proline and L- proline) and sugars
353 (sorbitol, sucrose, mannitol, glucose, dextrose, and dextran T-500), and others (hydrochloride
354 betaine and skimmed milk) have been used together with the cryoprotectants for macroalgal
355 cryopreservation (Jo et al., 2003; Lee and Nam, 2016).

356 Different cryoprotectants showed varied effects in different macroalgae species because cell
357 sizes, types of tissues, cell wall construction, and cytoplasm membrane composition. For
358 example, DMSO at 10% showed effective protection for *Ulva lobata* (highest post-thaw survival)
359 (Lalrinsanga et al., 2009), but was considered to be harmful to the fresh mature thalli and
360 germlings of *Ulva rigida* (Gao et al., 2017). Glycerol at 20% showed the highest protection
361 during cryopreservation for *Ulva prolifera* (Lee and Nam, 2016).

362 Cryoprotectants can be used independently or in combination for germplasm
363 cryopreservation (Taylor and Fletcher, 1999b). DMSO, the mostly used cryoprotectant for
364 macroalgae, is often combined with other cryoprotectants such as sugars and amino acids for
365 germplasm cryopreservation in many species (Tables 3, 4, and 5) (Kuwano et al., 1993; Kuwano
366 et al., 1994; 1996; Bhattarai et al., 2007; Zhou et al., 2007; Nanba et al., 2009; Choi et al., 2013;
367 Lee and Nam, 2016; Visch et al., 2019). Additionally, combinations of different cryoprotectants
368 (e.g., DMSO combining diglycerol; DMSO combing glycerol and proline) have been reported in
369 several studies with effective protection of germplasm materials were identified with high
370 post-thaw viability (Bhattarai et al., 2007; Choi et al., 2013; Lee and Nam, 2016).

371 The concentration of cryoprotectants is another important factor which affects the post-thaw
372 viability of cryopreserved germplasm. For example, high concentration of glycerol caused high
373 mortality of cryopreserved gametophytes of *Undaria pinnatifida* due to cytotoxic leakage of
374 vacuolar contents to the cell cytoplasm and destruction of thylakoids in plastids
375 (Ginsburger-Vogel et al., 1992). For macroalgae, the cryoprotectant concentrations used for
376 germplasm cryopreservation ranged from 5% to 25% (Tables 3-5). For example, the highest
377 post-thaw viabilities for male and female gametophytes of *Saccharina latissima* were found in
378 samples cryopreserved with DMSO 10% (v/v) using controlled-rate cooling methods (Visch et

379 al., 2019). Gametophytic thalli of *Ulva prolifera* had a viability of 91.6 % using a two-step
380 controlled-rate cooling method with 20 % glycerol (Lee and Nam, 2016). Optimal concentrations
381 varied depending on different cryoprotectants and macroalgal species. These conditions could be
382 determined through systematic evaluation of cryoprotectant toxicity on fresh cells and protection
383 during cooling process (examined on post-thaw viability).

384

385 *3.2.2. Equilibration time*

386 Before cooling, germplasm cells need to be mixed with cryoprotectants at optimal
387 concentration and equilibration time in culture medium to minimize intracellular water (Pegg,
388 2002). Overall, most publications used only one equilibration time, usually ranging from 15-60
389 min. Equilibration time used for macroalgae germplasm cryopreservation varied for different
390 species and germplasm materials, and even for the same species in different reports.

391 For *Gracilaria tikvahiae*, equilibration time between 5 to 90 min had no effects on
392 germplasm cell viability (van der Meer and Simpson, 1984). For *Neopyropia yezoensis,*
393 gametophytic thalli were found sensitive to cryoprotectants and a 5-second equilibration time
394 yielded the best post-thaw viability (Choi et al., 2013). The optimal equilibration time has been
395 investigated in several other macroalgal species, including the meiospores of *Saccharina*
396 *japonica* (Zhang et al., 2007b) and vegetative thalli of *Gracilaria corticata*, *Hypnea musciformis*,
397 and *Ulva lobata* (Lalrinsanga et al., 2009). For the meiospores of *Saccharina japonica*,
398 equilibration with 10% DMSO for 15 min was optimal (Zhang et al., 2007b). For vegetative
399 thalli of *Gracilaria corticata*, the equilibration time of 60 min was found to be the most optimal.
400 For vegetative thalli of *Hypnea musciformis* and *Ulva lobata* equilibration time of 45 min was
401 sufficient (Lalrinsanga et al., 2009). For (spore producing thallus) sporothalli filaments of
402 *Neoporphyra* and *Pyropia* species, the equilibration time of 45 min was found optimal (Jo et al.,
403 2003).

404 The temperature for the equilibration process can change the optimal equilibration time. For
405 macroalgae that inhabit temperate regions, such as *Ulva prolifera* and *Gracilaria tikvahiae*,
406 equilibration time was optimal at room temperature (usually 20-23 °C), while for macroalgae
407 that inhabit colder regions, such as *Neopyropia yezoensis* and *Saccharina latissima,* equilibration
408 was usually performed at lower temperatures at 0-10 °C (Zhou et al., 2007; Visch et al., 2019).

409 As mentioned above in this section, evaluation of acute toxicity at different temperatures could
410 be an effective approach to determine the optimal equilibration temperature.
411
412 *3.2.3. Mixing of cryoprotectants with germplasm materials*
413 The addition of cryoprotectants to germplasm materials could cause abrupt osmotic changes
414 because most cryoprotectants have high osmolarity. For example, DMSO has an osmolarity over
415 2000 mOsmol/kg. Therefore, the method of mixing cryoprotectants and germplasm materials is
416 critical to avoid sudden osmotic changes to germplasm cells. For macroalgae, cryoprotectants
417 were added gradually to macroalgae germplasm in majority of publications, including the
418 cryopreservation of conchocelis of *Neopyropia, Neoporphyra* and *Porphyra* spp. (*P. seriata*,
419 *Neopyropia yezoensis*, *Neopyropia tenera*, *Porphyra pseudolinearis* and *Neoporphyra dentata*)
420 (Kuwano et al., 1993; Jo et al., 2003), gametophytic cells of six species of Laminariales
421 (*Saccharina japonica*, *S. longissima*, *Kjellmaniella crassifolia*, *Ecklonia stolonifera*, *E. kurome*,
422 and *Undaria pinnatifida*) (Kuwano et al., 2004), vegetative thalli of *Gracilaria corticata*,
423 *Hypnea musciformis* and *Ulva* lobata (Lalrinsanga et al., 2009), gametophytic thalli of *Ulva*
424 *prolifera* (Lee and Nam, 2016), and gametophytes of *Undaria pinnatifida* (Nanba et al., 2009).
425 Generally, addition of cryoprotectants to germplasm materials were performed drop by drop
426 within 15 min, and the mixture was left for 45 min of equilibration time at specific temperatures.
427 Rapid mixing of cryoprotectants with target germplasm samples was also reported for
428 macroalgal cryopreservation, for example, gametophytic thalli of *Ulva rigida* (Gao et al., 2017),
429 gametophytes of *Saccharina latissima* (Visch et al., 2019), parthenosporophytes of *Ectocarpus*
430 (*E. siliculosus, Ectocarpus sp., E. fasciculatus*) (Heesch et al., 2012) and meiospores (suspended
431 or settled) of *Saccharina japonica* (Zhang et al., 2007b). Comparisons of mixing DMSO with
432 conchocelis cells of *Neopyropia yezoensis* with different times from 0-30 min revealed that
433 post-thaw survival was higher when DMSO was added gradually to reach its final concentration
434 than that when DMSO was added rapidly (Kuwano et al., 1993).
435 To compensate the osmotic change, cryoprotectant media in some studies were made in 50%
436 of seawater (van der Meer and Simpson, 1984) or even distilled water (Bhattarai et al., 2007). To
437 keep the pH balance after mixing of cryoprotectant and germplasm materials, HEPES (0.01 M,
438 pH = 8) was employed to make cryo-media in many studies (Kuwano et al., 1994; 1996; Jo et al.,
439 2003; Kuwano et al., 2004; Lalrinsanga et al., 2009; Nanba et al., 2009; Lee and Nam, 2016).

440 Similarly, the removal of cryoprotectant from post-thaw samples could be performed either
441 gradually or rapidly. The details were stated in section ***3.7***.
442
443 ***3.3. Packaging of macroalgal germplasm samples***
444
445 Packaging containers are important for germplasm cryopreservation because the cooling and
446 warming of germplasm samples is directly related to the volume, shape, and material type of
447 packaging containers. Currently, the commercially available containers include straws (0.25 ml,
448 0.3 ml, 0.5 ml, 5 ml) made of different materials (polyvinyl chloride for French straw, and
449 ionomeric resin for CBS™ straw) for sperm and embryos, cryopreservation bags for blood and
450 stem cells, cryovials made of different materials (polypropylene for the Corning™ cryovials) for
451 cell lines, and other custom containers.
452 For macroalgae germplasm cryopreservation, cryogenic vials (1.5 ml or 2 ml) were used in
453 almost all published studies as packaging containers (Tables 3, 4, and 5). Large volume cryovials
454 (5 ml) were sometimes used for germplasm cryopreservation of *Gracilaria corticata, Hypnea*
455 *musciformis*, and *Ulva lobata* (Lalrinsanga et al., 2009). Occasionally, straws (0.5-ml) were used
456 for macroalgal cryopreservation with high post-thaw viabilities, such as gametophytes and
457 meiospores of *Saccharina japonica* (Zhang et al., 2007b; Zhang et al., 2007a) and gametophytic
458 thalli of *Neopyropia yezoensis* (Choi et al., 2013). To date, no comparison of different packaging
459 containers has been reported for macroalgal germplasm cryopreservation.
460
461 ***3.4. Cooling process***
462
463 Based on the two-factor hypothesis (Mazur et al., 1972), cooling rate was considered a
464 critical factor of post-thaw cell viability. The optimal cooling rate could be empirically
465 determined depending on germplasm cell types, cryoprotectants, packaging containers etc.
466 (Mazur, 1977; Pegg, 2002). Cooling of samples can be conducted to a temperature (-40 °C or
467 -80 °C) at which samples and the cryo-medium should be completely frozen, and then frozen
468 samples can be directly immersed liquid nitrogen at -196 °C for long-term storage. This
469 approach is often called controlled cooling cryopreservation. Another method of
470 cryopreservation, called ‘vitrification.’ This process cools samples at ultrafast cooling rates,

471 which yield a glass-like ice transformation rather than ice crystallization to avoid injuring cells
472 during cooling process (Fahy et al., 1984). Vitrification has been successfully used for human
473 egg and embryo cryopreservation as routine for artificial fertilization (Loutradi et al., 2008;
474 Rienzi et al., 2017).
475
476 *3.4.1. Cryopreservation with controlled cooling rates*
477 For controlled cooling, a two-step cooling procedure was commonly applied for macroalgal
478 cryopreservation (Tables 3, 4, and 5). Cooling rates, initial temperature, final temperature, and
479 holding time of the first step and second-step cooling were different for different macroalgae
480 species. Overall, a controlled cooling rate at around 1 °C min^{-1} was applied for the first step
481 cooling procedure using a computer-programmed freezer or cooling at 1-6 cm above the surface
482 of liquid nitrogen. This probably related to the large cell size of macroalgal germplasm which
483 need more time for intracellular water transport during cooling process (Grout and Morris, 1987)
484 to avoid intracellular ice crystal formation (McLellan, 1989). For some macroalgal species such
485 as *Gracilaria tikvahiae*, slow and fast cooling rates (0.2 °C min^{-1} to 32 °C min^{-1}) did not yield
486 significant differences in post-thaw survival (van der Meer and Simpson, 1984). While in other
487 macroalgal species, such as *Saccharina japonica*, controlled cooling rates at 0.25 °C min^{-1} and
488 5.0 °C min^{-1} resulted in the loss of preserved meiospores. The post-thaw viability of meiospores,
489 immersed directly in liquid nitrogen, was 34% (Zhang et al., 2007b).
490 Initial temperature of the first step cooling process was usually the temperature for
491 equilibration of germplasm with cryoprotectants. For *Saccharina latissima,* a cold temperate
492 species, cooling started at 10 °C (Visch et al., 2019) while for *Ectocarpus* species (*E. siliculosus,*
493 *Ectocarpus* sp., and *E. fasciculatus*), which inhabit warm temperate regions, cooling started from
494 20 °C (Heesch et al., 2012). The final temperature of the first step cooling process was usually
495 −40 °C, which was demonstrated as optimal in majority of the reports for macroalgal germplasm
496 cryopreservation (Tables 3, 4, and 5). Cooling to a temperatures higher than −40 °C would
497 increase cell death because of insufficient intracellular dehydration (Kuwano et al., 1994). For
498 cryopreservation of gametophytes of *Saccharina japonica*, cooling of samples to -60 °C at a
499 cooling rate of 1 °C min^{-1} yielded a 59% post-thaw survival (Zhang et al., 2007a) .
500 Holding time between the first-step and second-step cooling procedure was different among
501 reports, e.g., 5 min for *Ulva prolifera* gametophytic thalli cryopreservation (Lee and Nam, 2016),

502 and 10~15 min for cryopreservation of gametophytes of *Saccharina latissima* (Visch et al.,
503 2019). The effect of holding times after the first step cooling on post-thawed gametophyte
504 viabilities was investigated in *S. japonica* and 40 min proved to be the optimal holding time
505 (Zhang et al., 2007a). In some reports, no holding time was reported after the first cooling step.

506 The second cooling step was to immerse samples directly into liquid nitrogen (-196 °C) or
507 liquid nitrogen vapor. Samples were then kept in liquid nitrogen (or vapor phase) for long-term
508 storage. This cooling procedure was reported in majority of macroalgae for germplasm
509 cryopreservation studies. However, for green macroalgae *Ulva intestinalis*, *Ulva fasciata*, *Ulva*
510 *pertusa*, and *Ulva rigida*, the second cooling step was to -80 °C rather than -196 °C. The reports
511 suggested high post-thaw survival for gamete and zoospore cryopreservation (Taylor and
512 Fletcher, 1999a; Bhattarai et al., 2007; Gao et al., 2017).

513

514 *3.4.2. Vitrification – ultrafast cooling*

515 Vitrification is the process of liquid "solidification" without ice crystallization but
516 transformation into a glass state (Harding et al., 2004). Complete vitrification requires ultra-fast
517 cooling rates and high sample viscosity by use of evaporative desiccation and/or employing
518 osmotic dehydration with penetrating cryoprotectants (Harding et al., 2004; Day, 2018). Another
519 method to achieve vitrification is encapsulation-dehydration, which involves in encapsulation
520 and dehydration of encapsulated materials by immersion and storage in liquid nitrogen (Sakai
521 and Engelmann, 2007). There are no toxic cryoprotectants required for samples, therefore
522 post-thaw samples can be cultured upon thawing without the need for cryoprotectant removal
523 (Taylor and Fletcher, 1999b).

524 Vitrification has been extensively applied to germplasm cryopreservation of animals, humans,
525 and higher plants (Harding et al., 2004; Sakai and Engelmann, 2007). To date, only a few studies
526 were reported on vitrification of macroalgal germplasm. Mostly vitrification was conducted by
527 simply immersing samples, with or without cryoprotectants, directly into liquid nitrogen (Tables
528 3, 4, and 5). For *Neopyropia yezoensis*, gametophytic thalli were vitrified by directly immersing
529 into liquid nitrogen after equilibration for 30 seconds with 5% DMSO and 15~25 % diglycerol,
530 yielded over 80% post-thaw viability (Choi et al., 2013). Haploid protoplasts were vitrified by
531 immediately immersing in liquid nitrogen after mixing with 25% vitrification solution (10% w/v
532 DMSO, 30% w/v glycerol, and 10% sucrose in seawater) and had a post-thaw viability as high as

533 66.5 % (Liu et al., 2004). For *Undaria pinnatifida*, gametophytes were vitrified in liquid nitrogen
534 by encapsulating into calcium alginate beads after mixing with 2 mol L^{-1} glycerol and 0.6 mol
535 L^{-1} sucrose for 90-120 min at 25 °C. Dehydration with vitrification solution (30% glycerol +15%
536 ethylene glycol +15% DMSO) for 40-50 min at 0 °C, and washing with 1.2 mol L^{-1} sucrose
537 solution, had a post-thaw viability of 26-31% (Wang et al., 2011). For *Scytosiphon lomentaria,*
538 filaments were vitrified after mixing with a solution (2 mol L^{-1} glycerol + 0.4 mol L^{-1} sucrose)
539 for 30 min. Dehydration followed with a vitrification solution (10 % DMSO + 10 % glucose +
540 10 % polyethylene glycol 6000) for 30 min at 0 °C. A rinse followed with 1.2 M sucrose for 20
541 min yielded a post-thaw survival rate of 38% (Zhuang et al., 2015).
542
543 ***3.5. Storage of cryopreserved germplasm samples***
544
545 Theoretically, cryopreserved samples need to be stored and maintained at temperatures
546 below -135 ºC/-140 ºC, the glass transition temperature. Commonly, liquid nitrogen or nitrogen
547 vapor were employed for cryopreserved sample storage in Dewars. Most evidence proved that
548 there was little or no detectable decline of viability of cells after many years storage at -196 °C
549 (Huang et al., 2019). Routine care of filling liquid nitrogen of the storage tanks needs to be taken.
550 Alternatively, electric ultra-freezers at -150 ºC can be used for storage of cryopreserved samples.
551 These ultra-freezers are commercially available, and many types have built-in liquid nitrogen
552 back-up systems, which can be self-activated if a power outage occurs.
553 Besides cryopreservation at -196 °C, germplasm samples could be occasionally preserved at
554 non-cryogenic temperatures (mostly at 4 °C) for a relatively short storage time for aquaculture
555 use, such as extending the growing season (Oohusa, 1984; Lobban and Harrison, 1994). For
556 example, thalli of *Neopyropia yezoensis* (1-3 cm) with 10-40% water content without
557 cryoprotectants was frozen and stored at -20 °C for 40 d, and survival after thawing was 93.10%
558 (Lin et al., 2010). Cultured blades of *Porphyra umbilicalis* (4.8 ± 0.22 mg) were directly frozen
559 for 1, 3, 6, or 12 months at –80 °C or -20 °C without cryoprotectants after being air-dried to 5 %
560 or 30 % absolute water content, and 100 % survival rates were obtained after thawing and
561 rehydration (Green and Neefus, 2014) (Table 1).
562
563 ***3.6. Thawing of cryopreserved samples***

564

565 For cryopreserved macroalgal germplasm, relatively few studies have been conducted on the
566 effects of thawing rate on the post-thaw survival (Tables 3, 4, and 5). Theoretically, factors
567 causing cell injury during cooling process would potentially cause cell injury during warming
568 process. Therefore, optimal thawing temperature or ultra-rapid warming rate (similar with
569 vitrification) needs to be determined.

570 Slow thawing has been used in recovery of non-cryogenic preserved macroalgal germplasm.
571 For example, thawing of blades of *Porphyra umbilicalis* preserved at -20 °C and -80 °C were
572 conducted by immersing into 125-mL of aerated sterile culture medium at 15 °C to recover for 3
573 h, and a 100% survival was achieved and continued to grow after rehydration (Green and Neefus,
574 2014). Different thawing temperatures (30, 40, and 50 °C) on the post-thaw survival of *Ulva*
575 *lobata*, *Gracilaria corticata* and *Hypnea musciformis* were tested, and thawing at 40 °C yielded
576 the highest post-thaw survival (Lalrinsanga et al., 2009). For *Neopyropia yezoensis* conchocelis,
577 thawing temperatures (20 °C, 30 °C and 40 °C) on post-thaw survival were tested, and again,
578 40 °C yielded the highest recovery rate of 87% (Zhou et al., 2007). In a majority of the published
579 studies, thawing of cryopreserved samples has been conducted merely by immersing the frozen
580 samples into a warm water bath at a temperature around 40 °C until samples had been
581 completely thawed.

582 Ultra-rapid warming has been emphasized in recent years to address the low post-thaw
583 survival especially for large-sized cells such as mouse embryos and oocytes after vitrification. It
584 was proposed that intracellular ice could be recrystallized during warming process and affect
585 post-thaw survival (Mazur and Paredes, 2016). With an infrared laser pulse and Indian ink at µm
586 sizes as heat-transfer medium, ultra-rapid warming at 10,000,000 °C/min was achieved.
587 Application on thawing of vitrified mouse oocytes yielded nearly 100% post-thaw survival (Jin et
588 al., 2014), even on thawing of vitrified mouse oocytes without permeable cryoprotectants (Jin and
589 Mazur, 2015). Application of ultra-rapid warming yielded a 10% post-thaw survival vitrified
590 zebrafish *Danio rerio* embryos (Khosla et al., 2017) and 43% in vitrified coral *Fungia scutaria*
591 larvae (Daly et al., 2018). Additionally, ultra-rapid warming was also achieved by using inductive
592 heating system and nanoparticles, and have been successfully applied on thawing vitrified tissues
593 and organs (Manuchehrabadi et al., 2017).

594 For macroalgae, the germplasm materials are usually large-sized, ultra-rapid or rapid
595 warming could have a significant effect on post-thaw survival. Rapid thawing of *Undaria*
596 *pinnatifida* gametophytes yield a 50-100% survival, which was significantly higher than that
597 from slow warming (Renard et al., 1992; Taylor and Fletcher, 1999b). Further investigation is
598 warranted for application of ultra-fast warming.
599
600 ***3.7. Post-thaw sample amendments***
601
602 One of the common post-thaw sample amendments was to remove the cryoprotectants in
603 post-thaw samples to avoid the toxicity of cryoprotectants on germplasm cells or tissues. This
604 amendment has been applied for human, livestock, poultry, and fish sperm cryopreservation
605 (Elliott et al., 2017). For macroalgae, removal of cryoprotectant in post-thaw samples has been
606 reported in majority of the publications before conducting viability assays. The first approach to
607 remove cryoprotectants was to dilute post-thaw samples with culture medium, for example, the
608 vegetative thalli of *Gracilaria corticata*, *Hypnea musciformis* and *Ulva lobata* (Lalrinsanga et al.,
609 2009). The gametophytic thalli of *Ulva prolifera* (Lee and Nam, 2016), the gametophytes of
610 *Undaria pinnatifida* (Nanba et al., 2009) and the gametophytic thalli of *Neopyropia yezoensis*
611 (Choi et al., 2013) required removal of cryoprotectants as well. The second approach to remove
612 cryoprotectants was to centrifuge the post-thaw sample followed by discarding of the supernatant.
613 This approach has been applied on conchocelis cells of *Neopyropia yezoensis* (Zhou et al., 2007)
614 and free-living conchocelis filaments of five species of *Neopyropia* and *Porphyra* (Jo et al.,
615 2003). So far, no differences were found in post-thaw viabilities between post-thaw macroalgal
616 samples with or without removal of cryoprotectants in post-thaw samples.
617
618 **4. Viability assays of post-thaw samples**
619
620 Easy, quick, and accurate viability assays are essential for development of cryopreservation
621 protocols. For macroalgae, post-thaw samples require several days to weeks to germinate or
622 grow into mature thalli for viability determination. A summary of the most widely methods for
623 viability assay in macroalgae germplasm is listed in Table 6. Based on the macroalgal cell

624 characteristics, the three commonly used viability assays were cell staining, measurement of cell
625 pigmentation index, and evaluation of re-growth.
626
627 ***4.1. Cell staining***
628 The stains for determination of living macroalgal cells include 0.05 % (w/v) erythrosine
629 (Nanba et al., 2009; Lee and Nam, 2016), 0.1% (w/v) fluorescein diacetate (Zhang et al., 2007b;
630 Zhang et al., 2007a), and 0.02-0.1 % (w/v) neutral red (Wang et al., 2000; Zhou et al., 2007).
631 After staining for about 20~30 min, macroalgal samples were rinsed with fresh seawater, and
632 observed immediately by use of light microscopy. The stained cells were determined as living
633 cells, while un-stained cells were dead cells. This method is easy to conduct, quick, and effective,
634 but may over-estimate the percentage of living cells. To date, neutral red staining is the most
635 widely used assay for macroalgae cell viability.
636
637 ***4.2. Measurement of cell pigmentation index***
638
639 Measurement of cell pigment index is another fast method for macroalgal viability assay
640 (Table 6), which was called the method of damage assessment (van der Meer and Simpson,
641 1984). Small pieces of thalli damaged by freezing lose all or part of their pigmentation and this
642 observation was used to devise a 'pigmentation index' where fully pigmented thalli were given a
643 score of 10 whereas completely white, dead thalli received a score of 0. Partially damaged thalli
644 were given intermediate scores reflecting both the area of dead tissue and the degree to which
645 surviving tissue had lost pigmentation. The pigments for evaluation include chlorophyll levels
646 (van der Meer and Simpson, 1984; Wang et al., 2005; Wang et al., 2011) and the ratio of viable
647 cells with brown color out of total cells for Ochrophyta macroalgae (Zhang et al., 2008; Visch et
648 al., 2019). The effectiveness of this method was confirmed for seaweed survival estimates
649 (Lalrinsanga et al., 2009; Gao et al., 2017).
650
651 ***4.3. Evaluation of re-growth***
652
653 The re-growth of post-thaw samples would be the gold standard for viability assay. This
654 viability assays were extensively used in majority of studies (Table 6). Comparison of this direct

655 method with other indirect methods (staining and pigment index) showed similar results,
656 indicating the effectiveness of indirect methods for viability assays (Leeson et al., 1984).
657 However, whenever time allows, the re-growth method should be employed for viability assays
658 of post-thaw macroalgal samples.
659

660 ### *4.4. Other viability assays*

661

662 Additional methods for macroalgal viability assay included the observation of ultrastructural
663 damage, liberation of spores or gametes, photosynthetic efficiency (PSII), and evaluation of
664 gamete formation. For macroalgal spores or gametes, germination rates would be the gold
665 standard for viability assay immediately after thawing (Taylor and Fletcher, 1999a; Bhattarai et
666 al., 2007; Gao et al., 2017).
667

668 Overall, any methods stated above could potentially be used for macroalgal viability assays
669 (Taylor and Fletcher, 1999b). However, large variation in viability may exist among these
670 viability assay methods (McLellan et al., 1991). Therefore, extreme caution should be exercised
671 when comparing the viabilities estimated by different methods among different publications.
672 Furthermore, even with the same viability assay, comparison of results from different
673 publications needs to pay attention to the definition of viability.
674

675 ## 5. Outlook and future research

676

677 Overall, macroalgal germplasm cryopreservation has been studied mostly in economically or
678 ecologically important species. Most of these studies were conducted empirically, and limited
679 application has been reported on the establishment of germplasm repository or commercial
680 macroalgal production. The majority germplasm types used for cryopreservation were
681 sporophyte or gametophyte thalli. Post-thaw viability showed significant variations among
682 different publications even for the same species. Depending on specific germplasm type and
683 studied species, the cryopreservation protocols showed variable results (Tables 3-5).
684

685 Based on the review of the current research updates, further investigation topics on
686 macroalgal germplasm cryopreservation should include:

687 1) Standardization of research protocols need to be improved through systematic evaluation of
688 some factors, such as addition of proteins, lipids, vitamins, or antioxidants to cryo-medium;
689 osmolality and pH change of cryo-medium; equilibration time and interaction with
690 cryoprotectants. Standardization of protocols would allow direct comparison of results from
691 different publications and could provide easy operation and assure quality control for the
692 application of macroalgal cryopreservation protocols;
693 2) Different packaging containers with potential for high-throughput processing need to be
694 evaluated and compared on protocol development for macroalgal germplasm repositories;
695 3) Research cryopreservation protocols for different types of germplasm need to be developed
696 including thalli (gametophytes or sporophytes), spore type (flagellated or amoeboid), and
697 gametes (isogametes, anisogametes or oogametes). Cryopreservation of spores and gametes
698 could facilitate preservation of numerous strains and species of macroalgae with less
699 requirement of labor and space;
700 4) Vitrification technology, such as encapsulation-dehydration with no toxin cryoprotectant,
701 needs to be developed for cryopreservation of macroalgal germplasm with direct use of
702 post-thaw samples without repeated washing; and
703 5) Use of ultra-rapid warming technology showed promising improvement on post-thaw
704 viability of large-cell or tissue vitrification (see Section 3.6). There is a potential that
705 ultra-rapid warming could be applied on macroalgal cryopreservation or vitrification.
706
707 Overall, germplasm cryopreservation for marine macroalgae have been majorly focused on
708 development of research protocols in the laboratory. These research protocols could be applied to
709 establish macroalgal germplasm repositories for commercial aquaculture and natural resources
710 conservation. Establishment of germplasm repository (seed banking) requires reliable research
711 protocols, infrastructure, repository management strategy for sample storage and inventory,
712 database management for phenotype, genotypes, and germplasm collection information, and
713 policy for use of cryopreserved samples.
714

Table 1. Summary about studies on macroalgal frost resistance or non-cryogenic cold storage. The species names used in original publications were annotated in parathesis following the new species names.

Species	Study Topic	Reference
Chlorophyta		
Monostroma angicava	Frost resistance for 24 hr to -20°C with 50% survival	(Terumoto, 1964)
Ulothrix flacca	Frost resistance for 24 hr to -25°C with 50% survival	(Terumoto, 1964)
Ulva intestinalis (*Enteromorpha intestinalis*)	Frost resistance by cooling to -5 to 25°C. Tolerance of -20°C for 24 hr	(Terumoto, 1961)
Ulva linza (*Enteromorpha linza*)	Frost resistance for 24 hr to -20°C with 50% survival	(Terumoto, 1964)
Ulva pertusa	Frost resistance by cooling from 0°C to -28°C. Tolerance of -10°C for 24 hr	(Terumoto, 1960; 1961)
Ochrophyta		
Fucus edentatus	Cold resistance of zygotes and embryos down to -25°C for hours and days	(Bird and McLachlan, 1974)
Macrocystis pyrifera	Cold storage of gametophytes at 10°C for 5 years	(Barrento et al., 2016)
Rhodophyta		
Bangia fusco-purpurea	Frost resistance for 24 hr to -55°C with 50% survival	(Terumoto, 1964)
Gloiopeltis furcata	Effects of different size, water content, and density on storage at 4°C and –18°C)	(Chen et al., 2016)
Neopyropia tenera (*Porphyra tenera*)	Frost resistance of vegetative thalli, spores, and *conchocelis* filaments	(Migita, 1964; 1966; 1967)
Neopyropia yezoensis (*Porphyra yezoensis*)	Frost resistance for 24 hr to -35 °C with 50% survival	(Terumoto, 1964)
	Effects of cooling rates and water content on frost resistance	(Migita, 1966)
	Freezing of thalli at -20°C after dehydrated with 10%–40% H_2O	(Lin et al., 2010)
Phycocalidia suborbiculata (*Porphyra suborbiculata*)	Effects of cooling rates and water content on frost resistance	(Migita, 1966)
Porphyra umbilicalis	Freezing to -20 °C or -80 °C after air dry to 5 or 30% H_2O (no cryoprotectants)	(Green and Neefus, 2014)
Pyropia pseudolinearis (*Porphyra pseudolinearis*)	Frost resistance for 24 hr to -55 (female) to -70°C (male) with 50% survival	(Terumoto, 1964)
Uedaea onoi (*Porphyra onoi*)	Frost resistance for 24 hr to -10°C with 50% survival, fatal at -15°C	(Terumoto, 1964)

Table 2. Summary about studies on germplasm cryopreservation in macroalgae. The species names in original publications were annotated in parathesis following the new species names.

Species	Study Topic	Reference
Chlorophyta		
Ulva intestinalis (*Enteromorpha intestinalis*)	Crypreservation of zoospores to –20°C to –40°C with DMSO and glycerol	(Taylor and Fletcher, 1999a)
Ulva fasciata	Cryopresrevation of spores at 4°C, -20 or -70°C with DMSO and glyerol	(Bhattarai et al., 2007)
Ulva lactuca	Application of cryopreservation protocol developed for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Ulva lobata	Cryopreservation of vegetative thalli with 10% DMSO or glycerol	(Lalrinsanga et al., 2009)
Ulva pertusa	Cryopresrevation of spores at 4°C, -20°C and -70°C with DMSO and glyerol	(Bhattarai et al., 2007)
Ulva prolifera	Cryopreservation of gametophytic thalli with DMSO, glycerol, or proline	(Lee and Nam, 2016)
Ulva rigida	Cryopreservation of thalli, germlings, and gametes for up to 184 days	(Gao et al., 2017)
Ochrophyta		
Ecklonia kurome	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Ecklonia stolonifera	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Ectocarpus fasciculatus, E. siliculosus E. sp.	Cryopreservation of different strains with DMSO 10% (v/v) and sorbitol 9%	(Heesch et al., 2012)
Eisenia bicyclis	Development of cryopreservation protocol	(Kono et al., 1998)
Kjellmaniella crassifolia	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Laminaria digitata	Vitrification by encapsulation dehydration	(Vigneron et al., 1997)
Saccharina japonica (*Laminaria japonica*)	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
	Cryopreservation of spores and gametophytes	(Zhang et al., 2007b; Zhang et al., 2007a)
	Cryopreservation of gametophytes using encapsulation-dehydration	(Zhang et al., 2008)
Saccharina longissima (*Laminaria longissima*)	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
Saccharina latissima	Development of cryopreservation protocol for gametophyte males and females	(Visch et al., 2019)
Scytosiphon lomentaria	Cryopreservation of filaments to -20°C through a vitrification procedure	(Zhuang et al., 2015)
Undaria pinnatifida	Development of cryopreservation protocol for six species of Laminariales	(Kuwano et al., 2004)
	Ultrastructure observation of gametophytes during thawing process	(Ginsburger-Vogel et al., 1992)
	Effects of pre-incubation irradiance on post-thaw survival	(Nanba et al., 2009)
	Cryopreservation of gametophytes by encapsulation-dehydration	(Wang et al., 2005; Wang et al., 2011)
	Development of cryopreservation protocol for gametophytes	(Arbault et al., 1990; Renard et al., 1992)
Vaucheria sessilis	Elucidation of the metabolic and structural basis during cooling process	(Fleck et al., 1999)
Rhodophyta		
Chondrus crispus	Application of cryopreservation protocol developed for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Devaleraea ramentacea	Application of cryopreservation protocol developedfor *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Gracilaria corticata	Cryopreservation of vegetative thalli with 10% DMSO or glycerol	(Lalrinsanga et al., 2009)
Gracilaria foliifera	Application of cryopreservation protocol developed for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Gracilaria tikvahiae	Development of cryopreservation protocol, and application on other five species	(van der Meer and Simpson, 1984)
Hypnea musciformis	Cryopreservation of vegetative thalli with 10% DMSO or glycerol	(Lalrinsanga et al., 2009)

Neoporphyra dentata (*Porphyra dentata*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994)
	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)
Neoporphyra haitanensis (*Porphyra haitanensis*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994)
	Cryopreservation of conchocelis using encapsulation-dehydration vitrification	(Wang et al., 2000)
Neoporphyra seriata (*Porphyra seriata*)	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)
Neopyropia tenera (*Porphyra tenera*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994; 1996)
	Development of cryopreservation protocol for sporothalli	(Migita, 1964; Jo et al., 2003)
Neopyropia yezoensis (*Porphyra yezoensis*)	Development of cryopreservation protocols gametophytic thalli	(Kuwano et al., 1996)
	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)
	Development of cryopreservation protocol for conchocelis	(Kuwano et al., 1992; Kuwano et al., 1993; Kuwano et al., 1994; Zhou et al., 2007)
	Vitrification of gametophytic thalli and sporrothalli	(Liu et al., 2004; Choi et al., 2013)
Palmaria palmata	Application of cryopreservation protocol for *Gracilaria tikvahiae*	(van der Meer and Simpson, 1984)
Porphyra linearis	Development of cryopreservation protocol for conchocelis	(Arbault and Delanoue, 1994)
Pyropia pseudolinearis (*Porphyra pseudolinearis*)	Cryopreservation of conchocelis cells by use of a Styrofoam box	(Kuwano et al., 1994)
	Cryopreservation of sporothalli with 10% DMSO and 0.5 M sorbitol	(Jo et al., 2003)

Table 3. Summary about germplasm cryopreservation in Chlorophyta macroalgae (7 species, 6 publications). The species *Enteromorpha intestinalis* in original publications was changed into *Ulva intestinalis*. Note: DMSO: dimethyl sulfoxide; EG: ethylene glycol; PG: propylene glycol, and PVP: polyvinylpyrrolidone.

Species	Germplasm	Cryoprotectant Agent	Cooling Process	Packaging container	Thawing temperature (°C)	Post-thaw viability	Reference
Ulva intestinalis	Settled zoospores	DMSO (5 and 10%) and glycerol (5 and 10%)	–1°C min^{-1} to –20°C or –40°C; –0.5°C min^{-1} to –20°C, and –1°C min^{-1} to -30°C, then in -196°C	None. Spores were on cover slips	Plunging the cover slips directly into VS culture medium at 37◦C	> 40% in samples frozen in 75% seawater at -20°C for 5 weeks	Taylor and Fletcher, 1999a
Ulva intestinalis	gametophytic thalli (< 5 mm)	Dextran, DMSO, PVP, proline, glycerol, PG, EG separated or combined with sorbitol, glucose, or sucrose	–1°C min^{-1} to –20, –30, -40, -50, and -60°C, and then in liquid nitrogen or not.	2-ml cryovial with 1.5 ml of samples	In water bath at 40°C until just before the ice melted	DMSO 10% showed best protection and -40°C was the best temperature. Post-thaw growth and gamete release were observed.	Kono et al., 1997
Ulva fasciata *Ulva pertusa*	Suspended spores	DMSO: 5%, paraffin oil, glycerol: 5%-20%. Separated or combined	Treated spores were preserved at room T, 4°C, –20°C, or –70°C. Cooling rates were not stated	Micro tubes, no volume stated	Resuspended in 1 mL of f/2 culture medium and incubated for germination in 24-well plates at 18°C	0-3% germination for samples preserved at -20°C, and 0 for sample at -70°C	Bhattarai et al., 2007
Ulva lactuca	Sporelings and apical segments	1.5 M DMSO	2°C min^{-1} to -40°C, then into liquid nitrogen -196°C	1-ml freezing ampoules 1 ml	36°C	100%	Van der Meer and Simpson, 1984
Ulva lobata	Apical tips (1-2 mm)	DMSO, EG, glycerol at 5%, 10%, and 15%	<-1°C min^{-1} to –20°C, –30°C, –40°C, –50°C, –60°C, then -196°C	4-ml sample in 5-ml cryovial	At 20°C, 30°C, 40°C, 50°C, and 60°C; The best thawing temperature was 40°C	8-29% at days 1-70	Lalrinsanga et al., 2009
Ulva prolifera	Gametophytes	DMSO, glycerol, and proline at 5, 10, 15, 20, or 25 %, separated or combined	At a cooling rate of 1°C min^{-1} from 15°C to -40°C, held at -40°C for 5 min, and then into liquid nitrogen	1.5-ml cryovial	40°C in water bath	92 % in post-thaw samples frozen with 20 % glycerol for 120 d	Lee and Nam, 2016
Ulva rigida	Gametophytes, germlings, and gametes	DMSO at 10 and 15% for thalli and germlings and 5 and 10% for gametes	1) Direct move to -20°C or -80°C 2) At 1°C min^{-1} to from 20°C to -20°C, then move to -80°C	1.5-ml freezing ampoules	Plunging the ampoules in a 37°C water bath	0% for gametophytic thalli at day 30; 0% for germling at day 1, and 4-19% for gametes at day 180	Gao et al., 2017

Table 4. Summary about germplasm cryopreservation in Ochrophyta macroalgae (14 species, 15 publications). The species *Laminaria japonica, Laminaria longissimi, and Kjellmaniella crassifolia* in original publications were changed into *Saccharina japonica, Saccharina longissimi,* and *Saccharina crassifolia.* Note: DMSO: dimethyl sulfoxide; EG: ethylene glycol; PG: propylene glycol.

Species	Germplasm	Cryoprotectant Agent	Cooling Process	Packaging container	Thawing temperature (°C)	Post-thaw viability	Reference
Ectocarpus siliculosus *Ectocarpus* sp. *Ectocarpus fasciculatus*	Sporophytes	10% DMSO and 9% sorbitol	1 °C min^{-1} from 20°C to -40 °C, then -196°C	1-ml sample in 2-ml Cryovials	40°C	25-50% for *Ectocarpus siliculosus*; >50% for *E.* sp. and *E. fasciculatus*	Heesch et al., 2012
Fucus edentatus	Zygotes and embryos	NA	Cooled to -2, -5, -10, and -15°C for 2 hr (zygotes and embryos); to -25°C (embryos)	NA	NA	34-92% for zygotes at -10°C; nearly 100% for post-thaw embryos.	Bird et al., 1974
Eisenia bicyclis	Gametophytes	EG and 10% proline	Pre-freezing temperature was -40°C, and then -196°C	N/A	N/A	52.5% and 62.0% after thawing, 31.1% and 27.2% after 4 d post-thaw culture	Kono et al., 1998
Laminaria digitata	Gametophytes	0.3-0.5M sucrose	Slowly from 19°C to -40°C and then -196°C.	N/A	40°C for 2 min	25-75%	Vigneron et al., 1997
Laminaria diabolica	Gametophytes	10% DMSO and 0.5M sorbitol	Slowly <1 °C min^{-1} to -40°C and then in liquid nitrogen.	2-ml vial with 0.75-ml sample	40°C	Recovery growth was found	Sakanishi and Saga, 1994
Saccharina japonica *Saccharina longissimi* *Saccharina crassifolia* *Ecklonia stolonifera* *Ecklonia kurome* *Undaria pinnatifida*	Gametophytes	EG, glycerol, DMSO, L-proline, sorbitol, sucrose, and dextran T-500	Slowly cooling to –40°C in 4 h, and then -196°C.	1.5-ml sample in 2-ml cryovial	40°C water bath and move to ice bath before melting.	3.1-73.3% for after thawing and 0-66.7 % for after 4 d post-thawing culture	Kuwano et al., 2004
Saccharina japonica	Gametophytes	10% EG and 10% proline	At 0.5, 1.0, 2.0 °C min^{-1} cooled to -30, -60, -90°C and holding for 0, 40, 80 min, then -196°C	0.5-ml straws	26°C water bath, then ice-bath before complete melting	69-84%	Zhang et al., 2007a
Saccharina japonica	Spores	DMSO, glycerol sorbitol, sucrose, and dextrose. Separated or combined	Direct cooling to -196°C; to -20°C within 30 min; at 0.25–5.0°C min^{-1} to -60°C, then -196°C (straw only).	1.5-ml cryovial and 0.5-ml straws	26 or 39°C water bath, transfer to ice-bath before complete melting	13-50%	Zhang et al., 2007b
Saccharina japonica	Gametophytes	0.4 M sucrose for 6 h	From 10°C to -40 or -60°C within 30 min, then -196°C	cryotubes	40°C	22-43%	Zhang et al., 2008
Saccharina latissima	Gametophytes	DMSO, D-sorbitol PG, and methanol. Separated/combined	1°C min^{-1} to -40°C or -80°C, then -196°C	1-ml sample in 2-ml cryovial	40°C water bath	Up to 80% for male gametophytes and 20% for female.	Visch et al., 2019
Undaria pinnatifida	Gametophytes	Glycerol at 5-10% or 28%; DMSO	Cooled to -80°C or -196°C	NA	NA		Arbault et al., 1990
Undaria pinnatifida	Gametophytes	Glycerol at 5-30%	5°C min^{-1} from 22°C to -30°C or -40°C; Directly in -196°C	NA	Rapid and slow thawing	Rapid thawing showed higher post-thaw viability than slow thawing	Renard et al., 1993
Undaria pinnatifida	Gametophytes	Glycerol at 5, 10, 15, and 20%	5°C min^{-1} to -40°C, then -196°C	2-ml cryovial	32°C water bath	NA. Ultrastructure was observed.	Ginsburger-Vogel, 1992
Undaria pinnatifida	Gametophytes	0.2-0.3 M sucrose for 9 h to dehydrate beads	Directly plunging at -196°C	2-ml cryovials	40°C for 2 min	7.3%-30.7 %	Wang et al., 2005
Undaria pinnatifida	Gametophytes	10% L-proline and 10% Glycerol	<1 °C min^{-1} to – 40°C in 4 h	2-ml Cryovials	40°C	43–100%	Nanba et al., 2009

Table 5. Summary about germplasm cryopreservation in Rhodophyta macroalgae (12 species, 13 publications). The new Genus *Pyropia* was used for *Porphyra pseudolinearis, Porphyra seriata,* and *Porphyra umbilicalis,* Genus *Neoporphyra* was used for *Porphyra haitanensis* and *Porphyra dentata*, and Genus Neopyropia was used for *Porphyra tenera* and *Porphyra yezoensis.* Note: DMSO: dimethyl sulfoxide; EG: ethylene glycol; PEG: polyethylene glycol; PG: propylene glycol.

Species	Germplasm	Cryoprotectant Agent	Cooling Process	Packaging container	Thawing (°C)	Post-thaw viability	Reference
Gloiopeltis furcata	Germling	None	Direct preservation at 4°C or -18 °C	0.5 g per Sealed bag	12 °C, and 16 °C for cultivation.	0% (4°C), and 16-72% (-18°C)	Chen et al., 2016
Gracilaria corticata	Apical tips (1-2 mm)	DMSO, EG, glycerol at 5%, 10%, and 15%	<-1°C min^{-1} to –20°C, –30°C, –40°C, –50°C, –60°C, then -196°C	4-ml sample in 5-ml cryovial	20, 30, 40, 50, and 60°C. 40°C was the best.	59%-85% at day 1-70	Lalrinsanga et al., 2009
Gracilaria tikvahiae	Sporelings and apical segments	1.5 M DMSO	Slow cooling rate to -40°C, then -196°C	1 ml freezing ampoules 1 ml	36°C	60-100%	Van der Meer and Simpson, 1984
Hypnea musciformis	Apical tips (1-2 mm)	DMSO, EG, glycerol at 5%, 10%, and 15%	<-1°C min^{-1} to –20°C, –30°C, –40°C, –50°C, –60°C, then -196°C	4-ml sample in 5-ml cryovial	20°C, 30°C, 40°C, 50°C, and 60°C	5.9-28.9%	Lalrinsanga et al., 2009
Neoporphyra haitanensis	Conchocelis	None	Dehydration the encapsulated beads, then -196°C	2 ml cryogenic vials	40°C	47.7-66.1%	Wang et al., 2000
Neoporphyra dentata *Pyropia pseudolinearis* *Pyropia seriata* *Neopyropia tenera* *Neopyropia yezoensis*	Conchocelis filaments	DMSO, glycerol, EG, proline, hydrochloride betaine, skimmed milk, sucrose, glucose, sorbitol, and mannitol	-1°C min^{-1} to -40°C, then -196°C	1.5 ml of sample in 2-ml cryovials	40°C	54.6-70.9% using 10% DMSO plus 0.5 M sorbitol	Jo et al., 2003
Pyropia umbilicalis	Small blades	None	Direct preservation at –20 °C or –80 °C	1.7-mL centrifuge tubes	15 °C for removing 3 h	100%	Green and Neefus, 2014
Neopyropia yezoensis	Conchocelis	DMSO with EG, PEG, sorbitol, and sucrose	<-1°C min^{-1} to –20°C, –40°C, then -196°C	2-ml cryovials	20°C, 30°C and 40°C	60-86% when using 10% DMSO plus 0.5 M sorbitol	Zhou et al., 2007
Neopyropia yezoensis	Conchocelis	10% DMSO and 0.5 M sorbitol in 50% seawater	0.1-1°C min^{-1} from -20 to -80°C at 0.1-1°C min^{-1}, then -196°C	2 ml cryogenic vials	40°C	>60 %	Kuwano et al., 1993
Neopyropia yezoensis	Gametophytes (5–10 mm)	DMSO, diglycerol, glycerol, PEG, PG, propanediol, butanediol at 5-50% in combination	Directly plunged into -196 °C	0.5-ml straw	40°C water bath	> 60% with 5-25% glycerol or diglycerol plus 5% DMSO	Choi et al., 2013
Neopyropia yezoensis	Gametophytes	None	After dehydration, direct preservation at-20°C	Self-sealing plastic bags	10°C and 20°C	93.10% with water content of 10–40%	Lin et al., 2010
Neopyropia yezoensis *Neopyropia tenera* *Porphyra pseudolinearis* *Neoporphyra dentata*	Conchocelis	5-15 % DMSO and 0.5 M sorbitol	1. 1°C min^{-1} to -20 to -60°C, then -196°C 2. Holding for 1h at -40°C, then -196°C	2 ml cryogenic vials	40°C	38.4-77.9 %	Kuwano et al., 1994
Neopyropia yezoensis *Neopyropia tenera*	Gametophytes	5 or 10% DMSO, 5% dextran T-500, PVP K-30, Ficoll 400, PEG 6000, PG, EG, glycerol, sorbitol, sucrose, or glucose	1°C min^{-1} to -20 to -60°C, then -196°C	2 ml cryogenic vials	40°C	12-96% using dextran, PVP or Ficoll combined with DMSO	Kuwano et al., 1996

Table 6. Viability assays of macroalgae in germplasm cryopreservation studies.

Methods	Viability Assay	Reference
Staining and/or examination of cells for ultrastructural effects	Neutral red (1:10000)	Terumoto, 1960; 1961; 1964; 1965
	Neutral red (0.1%); Erythrosine (0.05%)	Kuwano et al., 1992, 1993, 1994, 1996, 2004
	Neutral red (0.02%)	Wang et al., 2000
	Neutral red (0.1%)	Jo et al., 2003
	Neutral red (0.1%)	Zhou et al., 2007
	Neutral red (0.1%)	Zhuang et al., 2015
	Cell ultrastructural examination	Migita, 1964; 1966
	Cell ultrastructural observation using double fixation with glutaraldehyde (4%)-osmium tetroxide (1%)	Ginsburger-Vogel et al., 1992
	Fluorescein diacetate (3.6×10^{-5} M)	Liu et al., 2004
	Fluorescein diacetate (0.1 %)	Zhang et al., 2007a,b
	Erythrosine (0.05%)	Nanba et al., 2009
	Erythrosine (0.05%)	Choi et al., 2013
	Erythrosine (0.05%)	Lee and Nam, 2016
Measurement of photosynthetic rate	Fluorescence/photosynthesis measurement	Dudgeon et al., 1989, 1990
	Chlorophyll fluorescence measurements	Lin et al., 2010
	Photosynthetic efficiency of PSII measurement	Green and Neefus, 2014
Ability of cell division, regeneration, and re-growth	Percentage regeneration after 6-week cultivation	Van der Meer & Simpson, 1984
	Re-growth rate	Renard et al., 1992
	Cell division and formation of gametophytic thallus	Kuwano et al., 1994
	Developmental capacity	Wang et al., 2005
	Thallus regeneration	Lalrinsanga et al., 2009
	Developing sporophytes determination	Wang et al., 2011
	Regrowth rate after 6-8-week cultivation	Heesch et al., 2012
	Regrowth rate after 6-week cultivation	Green and Neefus, 2014
	Regrowth rate after 1-month cultivation	Zhuang et al., 2015
	Regrowth determination	Barrento et al., 2016
	Regrowth rate after 7-day cultivation	Chen et al., 2016
Measurement of spore liberation / gamete release	Liberation of spores from conchocelis	Migita, 1967
	Gamete release	Vigneron et al., 1997
Measurement of cell pigmentation	Pigmentation index	Van der Meer & Simpson, 1984
	Pigmentation index	Vigneron et al., 1997
	Chlorophyll measurement	Wang et al., 2005
	Cells with brown color (living) to the total	Zhang et al., 2007a, 2007b, 2008
	Pigmentation index	Lalrinsanga et al., 2009
	Chlorophyll a level after 8-day incubation	Wang et al., 2011
	Pigmentation index	Gao et al., 2017
	Cells with brown color (living) to the total	Visch et al., 2019
Spore/gamete germination	Germination rate of zoospores	Taylor and Fletcher, 1999a
	Germination rate of spores	Bhattarai et al., 2007
	Germination rate of gametes	Gao et al., 2017
Rate of gamete formation	Rate of gametogenesis	Lee and Nam, 2016

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