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CRYOPRESERVATION OF *Phaeocystis antarctica*

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Abstract

A large number of clonal isolates of the prymnesiophyte *Phaeocystis antarctica* have been established at the Alfred Wegener Institute in Bremerhaven, Germany, to address questions on the genetic diversity and ecological response patterns to climate change. However, at present the wider scientific community cannot access these strains and their long-term conservation, (currently by serial transfer), cannot be assured. Cryopreservation could provide the solution to these issues, as it would guarantee the long-term security of this genetically and ecological invaluable collection. This study outlines the successful application of conventional approaches and the use of novel, combined non-penetrating and penetrating cryoprotective strategies that have been successfully applied to the different life-stages of this alga.

Keywords: *Phaeocystis antarctica;* cryopreservation; cryoprotectant

INTRODUCTION

The bloom-forming prymnesiophyte alga *Phaeocystis* has a worldwide distribution and profoundly influences the ecology and biogeochemistry of almost all marine ecosystems (17, 40, 41). Six species have been identified and all have several morphologically distinct life-stages (16, 32, 40, 49). All six species have star-like filaments, which are capable of producing scaly flagellates; although, only three species form colonies (40). The two cold water colony-forming species *P. pouchetii* in the Arctic and *P. antarctica* in the Southern Ocean are known to be key species within their habitats and have major impacts on trophodynamics, community composition and biogeochemistry (8, 18, 19). *Phaeocystis* has been extensively studied in terms of bloom dynamics (1, 4), life-cycle stages (36, 37, 39, 40, 41, 47), dimethyl sulphide (DMS) release (25, 26, 27, 42), grazing and viral attack (5, 35, 48), but much less is known about its basic ecophysiological performance, i.e. growth under varying abiotic conditions such as temperature, irradiation or salinity. The combination of traditional molecular methods with physiological growth experiments could lead to a detailed analysis of the population structure of both polar species and is intended to link genetic data to environmental parameters and to ecophysiological response patterns. "Ecophysiological genetics" is a novel approach to investigate *Phaeocystis* that offers new insights into ecological questions and the reaction of key species to predicted climatic changes in both polar regions (18, 19).

A polar algal collection comprising 230 clonal strains of *P. antarctica* and 400 clonal strains of *P. pouchetii* has been developed at the Alfred Wegener Institute (AWI) in Bremerhaven, Germany as a research tool to help address questions on their genetic diversity and ecological response patterns. At present these strains are maintained by routine serial transfer and resources to maintain them cannot be guaranteed beyond the life-time of the current project. Cryopreservation could ensure the long-term security and availability of this genetically/ ecological interesting collection, to the wider scientific community. However, the application of cryopreservation to algae has had variable levels of success, not least because of the ecological, taxonomic, morphological and functional diversity of this group (11, 21, 22, 38, 45).

In this study we have investigated the application of conventional colligative and novel combined non-penetrating/ penetrating cryoprotective strategies, as well as a non-penetrating cryoprotective strategy to cryopreserve different life-stages of *P. antarctica*.

MATERIALS AND METHODS

Organism and culture regime

Clonal cultures of *Phaeocystis antarctica* have the capacity to produce a range of different morphotypes including: colonial cells embedded in colonies or separate, flagellates and an attached aggregate (AA) stage (Fig. 1). The AAs consist of clumps of diploid, non-motile cells surrounded by a tough, sticky "skin" and it has been suggested that they might be able to undergo meiotic division to produce haploid micro- and mesoflagellates (17, 37).

In this study two morphologically representative strains of *P. antarctica* (Table 1) were cultivated/ maintained in GP5 medium (28), based on Antarctic-seawater in 25 ml tissue culture flasks (Corning, USA) at 2°C to 4°C under a 12h/12h light/dark regime, with photosynthetically active radiation levels (PAR) of 25 µmol m^{-2} s^{-1}.

Table 1. *Phaeocystis antarctica* cultures investigated in this study

Strain ID	Predominant cell stages in culture	Origin	Latitude	Longitude	Isolator
69/770_50	AAs & flagellates	Riiser-Larsen Sea	-68,01483	23,7705	Gaebler-Schwarz
69/905_100	colonies & colonial cells	Indian Ocean	-56,16017	76,44267	Gaebler-Schwarz

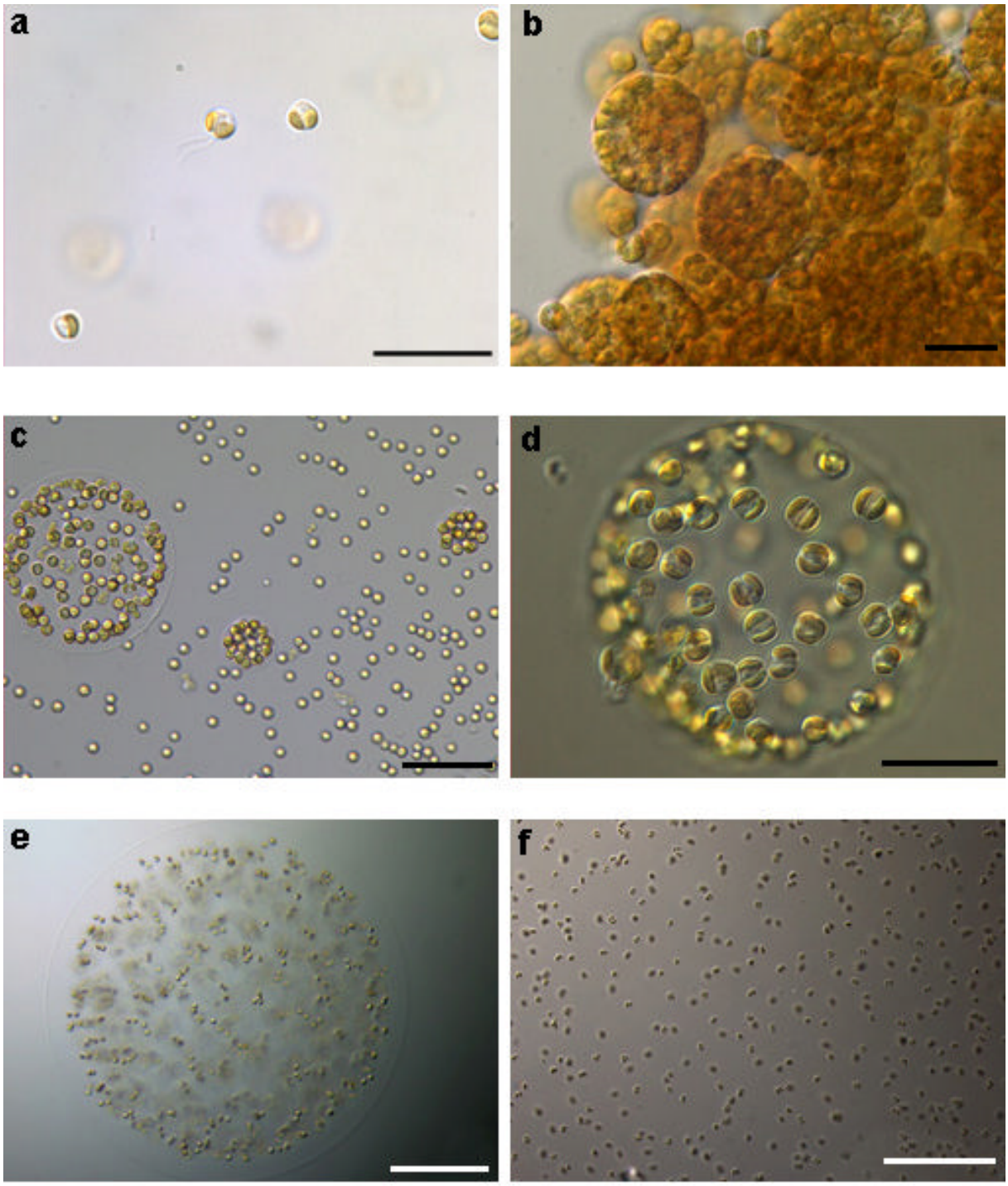

Figure 1. Overview of different *P. antarctica* morphotypes (developmental stages): a) flagellates; b) AAs; c) overview of various colonial stages; d) young colony; e) middle-sized colony; and f) large colony. (Scale bar for a, b, d = 20 µm; c = 50 µm, e, f = 100 µm), (adapted from 16)

Cryoprotective strategies

As outlined below, the following cryoprotective agents (CPAs) were prepared: dimethylsulfoxide (DMSO, 20% v/v, or 10% v/v in GP5 medium) and methanol (20% v/v, or 10% v/v in GP5 Medium). These were used singly, by aseptically combining equal volumes

of culture and CPA (0.5 ml) dispensed into 1.8 ml cryovials or in combination with other CPAs. Samples were then held on ice for 2 h prior to further cooling.

An alternative combined cryoprotection strategy was performed by inserting an additional step after the 2 h CPA incubation. Depending on which compound was employed, either 2.5 ml fish gel (FIB Foods BV, Netherlands) /GP5 (40%), or 2.5 ml methyl cellulose (Sigma, UK) /GP5 (3%) were added to 2.5 ml culture/cryoprotectant solution, mixed gently, prior to aliquots (1 ml) being dispensed into 1.8 ml cryovials.

Cooling procedure

A two-step cooling method was employed using a controlled-rate cooler (Kryo 360 3.3, Planer, UK) and subsequent plunging in liquid nitrogen (10), with minor modifications as outlined below:

1. The incubation time period, after addition of the CPA was increased from 20 min to 2 h.
2. Cooling was initiated at 0°C, cooling at -1°C min^{-1} to -40°C and held 15 min at -40°C prior to plunging into liquid nitrogen.
3. All culture manipulations and transfers were performed on ice, to ensure they did not experience elevated temperatures.

Rewarming procedure

After a minimum of 1 h in liquid nitrogen cryovials were thawed by placing them in a 40°C water bath. To ensure that sample temperatures did not exceed ~5°C, as the last visible ice melted samples were rapidly transferred to an ice-bath, prior to inoculation into tubes containing 5 ml GP5 medium (at 2°C).

Viability assessment of P. antarctica *cells using CFSE staining*

Cultures were maintained for 2 days in the dark at 2°C to 4°C, then uncovered and incubated under standard culture conditions (see above). Cell viability was assessed by microscopy employing the non-cytotoxic fluorescent vital stain carboxyfluorescein diacetate succinimidyl ester (CFSE) that allows discrimination between viable and non-viable cells (30). Aliquots of cell suspensions (1 ml) were used for viability staining with 2 μM CFSE [0.5 mM in 10% Me_2SO]. Three replicates were counted for each sample investigated. Cultures were monitored for survival/growth directly after the dark period and after 4 weeks incubation under light at 2°C to 4°C.

RESULTS

Conventional cryoprotectants

Vital staining employing CFSE allowed rapid assessment of post-thaw viability, with an obvious difference in signal being observed between CFSE positive cells (green) and red autofluorescence of chlorophyll in non-viable cells (Fig. 2). Four conventional cryoprotection treatments were tested: (i) 5% methanol, (ii) 10% methanol, (iii) 5% DMSO and (iv) 10% DMSO, on different cell stages of *P. antarctica*. No evidence of cryoprotectant toxicity was observed in either strain studied following short-term (1 h), or 24 h incubation. On subsequently cooling using 1°C min^{-1} to a terminal temperature of -40°C, followed by plunging into liquid nitrogen, no viability was observed in any samples pre-treated with 5% DMSO. However, for all other conventional cryoprotectant treatments vital-staining viability tests 48 h after thawing demonstrated survival of all cell types (Table 2). Despite apparent viability, on the basis of vital staining, cultures with colonies/colonial cells and flagellates

showed no growth after a period of four weeks, except for poor growth/survival of flagellates pre-treated with 10% methanol, whereas cultures with AAs and flagellates had good growth in thawed samples that had been pre-treated with 10% DMSO, or 10% methanol (Table 2).

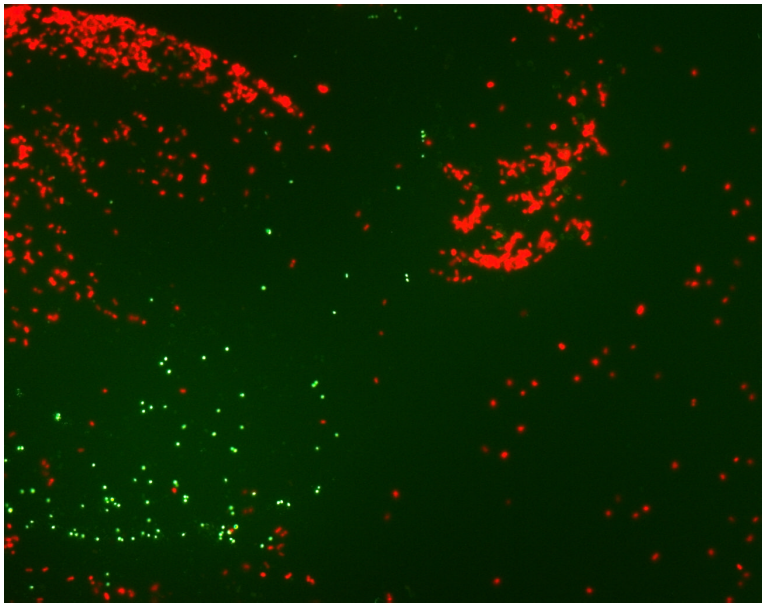

Figure 2. Post cryopreservation viability assessment of exemplar *P. antarctica* culture using CFSE

Table 2. Post-thaw viability[1] of *Phaeocystis antarctica* employing conventional cryoprotectants

	Isolate 69/905_100 (colonies)			Isolate 69/770_50 (AA)[2]		
Cryoprotectant	5% methanol	10% methanol	10% DMSO	5% methanol	10% methanol	10% DMSO
Colonies[1]	0%	87±23%	0%	n/a	n/a	n/a
AA[1,2]	n/a	n/a	n/a	33±58%	67±58%	33±58%
Flagellates[1]	47±10%	62±15%	41±19%	25±21%	16±11%	20±18%
Regrowth/live[3] colonies/AA	-	-	-	++	+	++
Regrowth/live[3] flagellates	-	+	-	-	++	++

[1]Mean viability levels ± standard deviation, assessed 48 h after thawing by CFSE staining and regrowth after 4 weeks under standard environmental conditions
[2]AA: Attached Aggregates
[3]Regrowth: – (cell death); + (survival of inoculum); ++ (active growth); +++ (good growth)

Combined penetrating and non-penetrating cryoprotectant strategy

Four novel, combined penetrating and non-penetrating cryoprotectant strategies were tested: (i) 5% methanol/methyl cellulose, (ii) 10% methanol/methyl cellulose, (iii) 5% methanol/fish gelatin and (iv) 10% methanol/fish gelatin on two types of cultures:

colonies/colonial cells with flagellates present and cultures comprised of AAs with flagellates. Viability assessment by vital staining 48 h after rewarming/thawing indicated high levels of survival of Isolate 69/905_100 cultures treated with 10% methanol/methyl cellulose, but no colony survival was observed in samples when treated with 10% methanol/fish gelatin (Table 3). The same trend was observed for Isolate 69/770_50 comprised of the AA cells stages (Table 3). Viability levels 48 h after thawing were not assessed for samples treated with 5% methanol, but survival may be inferred as after four weeks incubation under standard culturing conditions, good growth of samples treated with both 5 and 10% methanol with methyl cellulose was observed (Table 3). Although both strains survived and were capable of regrowth, it was noted that colonies in culture Isolate 69/905_100 on regrowth generated AAs, rather than standard colonies.

On employing methanol/fish gelatin as a cryotectant mixture, colony survival was observed in samples of Isolate 69/905_100 that had been treated with 5% methanol in combination with fish gelatin. Although despite the presence of CFSE positive cells 48 h after thawing in samples that had been treated with 10% methanol and fish gelatin no regrowth was observed (Table 3). Furthermore, no long-term recovery was observed for Isolate 69/770_50 samples treated with methanol/fish gelatin (Table 3).

Table 3. Post-thaw viability[1] of *Phaeocystis antarctica* employing a joint penetrating and non-penetrating cryoprotection strategy

	Isolate 69/905_100 (colonies)				Isolate 69/770_50 (AA)[2]			
Cryoprotectant	Methylcellulose		Fish gelatine		Methylcellulose		Fish gelatine	
	5% methanol	10% methanol	5% methanol	10% methanol	5% methanol	10% methanol	5% methanol	10% methanol
Colonies[1]	no data	91±9%	no data	0%	n/a	n/a	n/a	n/a
AA[1,2]	n/a	n/a	n/a	n/a	no data	57±13%	no data	5±9%
Flagellates[1]	no data	56±10%	no data	33±1%	no data	53±19%	no data	13±8%
Regrowth/live[3] colonies/AA	++[4]	++[4]	+	-	++	++	-	-
Regrowth/live[3] flagellates	-	-	-	-	++	++	-	-

[1]Mean viability levels ± standard deviation, assessed 48 h after thawing by CFSE staining and regrowth after 4 weeks under standard environmental conditions.
[2]AA: Attached Aggregates
[3]Regrowth: – (cell death); + (survival of inoculum); ++ (active growth); +++ (good growth).
[4]Colonies changed to AAs.
n/a: not applicable, because the strain does not normally produce colonies, or AAs respectively.
No data: samples not examined by CFSE staining

Preliminary studies using a single, non-penetrating cryoprotectant (fish gelatin), on isolates of *P. antarctica* have resulted in good levels of post-thaw viability (46 – 96%) on the basis of vital staining and all strains tested have regrown on transfer to fresh medium. Furthermore, satisfactory recovery was observed in samples cooled employing either a controlled rate cooler followed by transfer to liquid nitrogen, or by direct plunging of samples into liquid nitrogen.

DISCUSSION

The phenotypic, physiological, genomic and ecological diversity of algae present major challenges to the development of robust conservation methodologies. *P. antarctica* is a particularly problematic taxon to conserve as it is an obligate psychrophile, with a range of morphotypes. These factors need to be considered in terms of cryopreservation method development. In this study steps, such as cryoprotection, routinely performed at ambient temperature (9) were performed on ice. Additionally, it was possible that rewarming may be the critical stage of the cryopreservation process, so extreme care was taken to ensure that samples never exceeded 10°C, to avoid possible injuries.

The strains investigated produce a range of cell-types with different cells: colonial cells [~3.2µm to 10µm (31, 33, 40, 47)]; haploid flagellate [~2.4 to 7 µm (6, 7, 31, 43)]; diploid flagellate [~6.5 to 7.5 µm (15, 20, 40)]; and AAs [~4.2 to 9.8 µm in diameter (17)]. The different morphologies may result in varying susceptibility to cryoinjury, as it has previously been reported that in motile algae the flagellar insertion point is particularly susceptible to mechanical injuries during freezing and thawing (14). In this study viability levels for flagellate cells were usually much lower than for AAs or colonial cells (Tables 2 & 3), suggesting that they were more sensitive to the physical and chemical stresses induced by freezing and thawing.

Cryoprotection was crucial to the successful application of cryopreservation. Both methanol and DMSO, at the concentrations employed, had no obvious cytotoxic effects on the alga and have previously been successfully used for a wide variety of algae (9, 46). Without the addition of cryoprotectants no survival/ regrowth was observed in controlled-rate, or plunge cooled samples. For isolate 69/770_50 culture recovery reflected viability assessment by vital staining (Tables 2 & 3); however, for Isolate 69/905_100, despite high levels of viability on the basis of vital staining, poor regrowth was observed on the use of either methanol, or DMSO as cryoprotectant (Table 2). A further interesting observation was the change in post-thaw culture morphology in Isolate 69/905_100, where AAs rather than colonies were noted post-thaw. The authors have observed this phenomenon previously in culture and it could be speculated that AAs might be formed in response to the stresses experienced by the alga, as *in vivo* these may act as an overwintering stage (17, 37).

Ice-free vitrification-based cryopreservation is one of the main approaches employed for preserving plant germplasm (2) and has also been used for algal storage (21, 22). To facilitate vitrification, enhancement of cell viscosity is usually achieved by either the addition of cryoprotective additives at very high concentrations including high molecular-weight compounds that will not pass through the cell membrane, or alternatively by removal of water via evaporative desiccation and/or osmotic dehydration (2). Desiccation-based dehydration approaches have been successfully applied to a range of microalgae across European algal collections (12, 22, 29), but attempts to use this method were unsuccessful in this study as technical challenges to maintain the low temperature required to retain gelation in the fish gel (<5°C), whilst facilitating evaporative desiccation to the critical <15% residual moisture level (21, 34), could not be overcome. Standard chemical vitrification approaches, such as the

application of PVS2 can be injurious, as additives may be toxic to algae when applied at high concentrations, or result in osmotic-induced damage (3, 13). In this study alternative non-toxic agents (methylcellulose, or fish gelatin) were employed. Using a combined cryoprotectant strategy higher reliability, as evidenced by active post-thaw growth, was achieved than for experiments employing a single cryoprotectant (Tables 2 & 3). Historically, algae have been cryopreserved employing single cryoprotectants with DMSO or methanol being the cryoprotectant of choice (9); however, more recently cryoprotectant cocktails using a mixture of sorbitol and DMSO have proven to be very effective for the "model" brown alga *Ectocarpus* (23) and this approach warrants further investigation for a wider range of taxa.

The successful preliminary studies using a single, presumed non-penetrating cryoprotectant, on isolates of *P. antarctica*, in conjunction with direct plunge cooling in liquid nitrogen, has the potential to provide greater flexibility for the phycological community to utilize cryopreservation, as most researchers in the field do not normally have access to controlled rate coolers. This warrants further investigation.

The application of different types of cryoprotective strategies for algal cryobanking is clearly dependent upon the susceptibility of each organism, or cell type, to cryoinjury and the potentially deleterious effects of cryoprotection. The most widely applied approach - controlled rate cooling and colligative cryoprotection - requires an organism to survive chilling, extracellular freezing and osmotic stress. The ecological origin of the strains may have a profound influence on their capacity to survive these stresses and from the authors' experience, terrestrial isolates and those subjected to periodic stresses, such as littoral taxa are most amenable to conventional approaches. Vitrification, on the other hand, necessitates high tolerance to osmotic stress and desiccation injury (21). *P. antarctica* originates from the polar seas where surface water temperatures at the collection sites do not exceed 4.0°C in summer (24). Additionally, they may occasionally be periodically subjected to the hypersaline environments found in brine channels sea ice and prolonged periods of darkness during the austral winter (44). It would appear that this alga's capacity to survive natural environmental extremes assist it in coping with cryopreservation-induced stresses experienced on employing either a colligative or vitrification-based approach.

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