

# Cryopreservation of some useful microalgae species for biotechnological exploitation

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**Abstract** Culture collections of microalgae represent a biological resource for scientific research and biotechnological applications. When compared to the current methods of maintenance and sub-culturing, cryopreservation minimizes labor costs and is an effective method for maintaining a large range of species over long periods with high stability. In order to determine the best cryopreservation method for microalgae species with great biotechnological potential, three freezing protocols were employed using different cryoprotectants (dimethyl sulfoxide—Me<sub>2</sub>SO; methanol—MeOH). Three marine microalgae species (*Thalassiosira weissflogii*; *Nannochloropsis oculata*, and *Skeletonema* sp.) were cooled by directly plunging into liquid nitrogen (−196°C) and with two-step controlled cooling protocols (−18°C and −80°C pre-treatments). After storage periods ranging from 10 to 120 days, viability was determined by the ability of cells to actively grow again. Results obtained for *T. weissflogii* showed that this species could be preserved at ultra-low temperature (−196°C) for 10 and 30 days with 10% Me<sub>2</sub>SO and 5% MeOH when employed a controlled cooling protocol (−80°C). *N. oculata* was successfully cryopreserved either by direct freezing or with controlled cooling protocols. *N. oculata* samples presented good responses when treated with 5% Me<sub>2</sub>SO, 10% Me<sub>2</sub>SO, 5% MeOH and even

without any cryoprotectant. *Skeletonema* sp. did not survive cryopreservation in any of the tested conditions. The results indicate the difficulty in establishing common protocols for different microalgae species, being necessary further studies for a better understanding of cell damages during freezing and thawing conditions for each species.

**Keywords** Cryopreservation · Microalgae · *Nannochloropsis oculata* · *Thalassiosira weissflogii* · *Skeletonema* sp.

## Introduction

To maintain a culture of microalgae is a laborious activity, which consumes great deal of time. Therefore, researchers dedicated to collections of these microorganisms have a particular interest in the development of cryopreservation techniques (Lourenço 2006). Cryopreservation can be defined as the storage of organisms at ultra-low temperatures (less than −130°C), in a way that it still able to grow after thawing (Day and Brand 2005). Microalgae cultures kept alive in such arrested state are protected from genetic drift, contamination, and also reduce the cost of long-term maintenance (Brand and Diller 2004). However, due to the heterogeneity of microalgae species, progress in cryopreservation is gradual and depends on specific protocols for some strains. In spite of it, the interest in cryopreserved marine microalgae increases rapidly mainly because of their biotechnological potential (Rhodes et al. 2005).

Despite the advantages of cryopreservation, hundreds of species have not been successfully preserved under ultra-low temperatures. A range of freezing protocols have been developed for the cryopreservation of algae, though optimum methods appear to vary from species to species (Taylor

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and Fletcher 1999). In most cases, success in cryopreservation depends on the application of the appropriated rate of cooling, cryoprotective additives, osmotic equilibrium, and ice nucleation process. With respect to the freezing of marine microalgae, the high concentration of electrolytes and other solutes in the extracellular medium result in a deep dehydration during ice crystallization. Plasmolyzed cells experience severe mechanical deformation and intracellular solute concentrations may increase to toxic levels (Day and Brand 2005). These processes may explain the difficulty in cryopreservation of some marine species.

In this study, we tested the effectiveness of three freezing protocols and two cryoprotectants (dimethyl sulfoxide— $\text{Me}_2\text{SO}$  and methanol— $\text{MeOH}$ ) on the survival of three microalgae species (*Thalassiosira weissflogii*; *Nannochloropsis oculata*, and *Skeletonema* sp.) that present great potential for metabolites and biofuels production (Borges et al. 2007; Mata et al. 2010). Despite the fact that some freezing protocols were already developed for some of these microalgae species, we decided to test the effectiveness of freezing tools normally available in laboratories (liquid nitrogen; freezer; ultra-freezer; controlled-cooling canister—“Mr. Frosty”), in combination with common cryoprotectants, in order to elect the most suited procedure to cryo-storage each of the tested microalgae species.

## Material and methods

*Nannochloropsis oculata* (NANN OCUL-1), *Thalassiosira weissflogii* (THAL WEIS-1), and *Skeletonema* sp. (SKEL COST-1) strains were obtained from the Phytoplankton and Aquatic Microorganisms Laboratory of the Federal University of Rio Grande—FURG. The selection of microalgae species was according to their potential as feedstock for biodiesel production (Borges et al. 2007).

Strains were grown in glass culture tubes containing 20 mL of *f/2* medium (Guillard 1975) salinity (28 ppt). Cultures were incubated at 20°C with illumination of 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , provided by white fluorescent light lamps with a 12:12 h light/day cycle.

When cultures arrived the exponential phase (LOG), with cell density of approximately  $10^5 \text{ cell mL}^{-1}$ , 1 mL of each strain was re-inoculated in 20 mL of *f/2* medium and cell growth followed until a new LOG phase, when freezing experiments began.

### Cryoprotective solutions

A “half strength” (half concentration of all elements) *f/2* medium was used to prepare the cryoprotective solutions with dimethyl sulfoxide ( $\text{Me}_2\text{SO}$ ) and methanol ( $\text{MeOH}$ ) at the concentrations of 10% and 20% (v/v).  $\text{Me}_2\text{SO}$  was sterilized at 120°C for 30 min, whereas  $\text{MeOH}$  was

sterilized by filtration through a fiberglass filter (Whatmann—0.75  $\mu\text{m}$ ). An appropriate volume of cryoprotective stock solution was combined with an algal liquid culture to achieve the desired final concentration of cryoprotective agent (CPA). A treatment was submitted to the same freezing procedures in a “half strength” medium without any CPA addition in order to estimate potential natural freezing tolerance.

### Freezing protocols

Three freezing procedures were tested. The first procedure was the rapid cooling technique. It consists mostly of simply plunging the algal material rapidly into liquid nitrogen at  $-196^\circ\text{C}$  (Taylor and Fletcher 1999). Cryoprotective solution (1 mL of each) was added to an equal volume of microalgae culture (LOG phase) in 2 mL cryotubes, resulting in a CPA's final concentration of 5% and 10% (v/v). There was also a treatment without any cryoprotective agent. After 30 min acclimatization in the dark at room temperature, samples were directly transferred to liquid  $\text{N}_2$ .

The other cryopreservation protocols applied utilized a two-step cooling process with controlled cooling regime. A controlled-cooling canister, called “Mr. Frosty” (Nalgene®, Nalge Nunc International), containing isopropyl alcohol, was cooled to 4°C. This device allows a cooling rate of  $-1^\circ\text{C min}^{-1}$ . In both controlled cooling protocols, cryotubes containing algal cultures prepared for cryopreservation were placed into “Mr. Frosty” and kept in the dark at room temperature for 30 min to equilibration.

The basic difference between the two protocols is in the first step of temperature decrease. One procedure was determined by inserting “Mr. Frosty” with cryotubes into a freezer ( $-18^\circ\text{C}$ ) for 24 h until the samples reach  $-12^\circ\text{C}$ , being an adaptation of the protocol described by Brand and Diller (2004). In the third protocol, “Mr. Frosty” with samples was placed into an ultra-freezer ( $-80^\circ\text{C}$ ) for 1.5 h until the temperature had reached  $-50^\circ\text{C}$  (Brand and Diller 2004). After reaching the lowest temperatures in both cases, cryotubes were then immediately transferred to a liquid nitrogen storage system ( $-196^\circ\text{C}$ ) and kept there for different periods (see experimental design in Table 1).

### Thawing procedure

After storage periods ranging from 10 to 120 days, cryotubes were removed from liquid  $\text{N}_2$ , plunged directly in a preheated water bath ( $40^\circ\text{C}$ ) and gently agitated until the contents were fully melted (approximately 4 min). The thawed cultures were then transferred to glass culture tubes containing 20 mL fresh *f/2* medium to dilute the CPA.

After 24 h of dark incubation at room temperature, cultures were maintained in the growth conditions described previously, with the only difference that in the fourth

**Table 1** Experimental design

Experiment number	Microalgae species	Freezing procedure	Treatments	Cryo-storage period (days)	Growth conditions after thawing
1	<i>N. oculata</i> , <i>T. weissflogii</i> , <i>Skeletonema</i> sp.	Culture plunged directly into liquid N (−196°C)	Control 5% and 10% MeSo, 5% and 10% MeOH without CPA (WCPA)	10	Sal. 28 ppt, temp. 20°C, light 40 μmol photons m <sup>−2</sup> s <sup>−1</sup>
2	<i>N. oculata</i>	Pre-cooling −18°C, cryo-storage in liquid N (−196°C)	Control 5% and 10% MeSo, 5% and 10% MeOH, WCPA	10	Sal. 28 ppt, temp. 20°C, light 40 μmol photons m <sup>−2</sup> s <sup>−1</sup>
3	<i>N. oculata</i> , <i>T. weissflogii</i> , <i>Skeletonema</i> sp.	Pre-cooling −80°C cryo-storage in liquid N (−196°C)	Control 5% and 10% MeSo, 5% and 10% MeOH, WCPA	10	Sal. 28 ppt, temp. 20°C, light 40 μmol photons m <sup>−2</sup> s <sup>−1</sup>
4	<i>N. oculata</i> , <i>T. weissflogii</i> , <i>Skeletonema</i> sp.	Pre-cooling −80°C cryo-storage in liquid N (−196°C)	Control 5% and 10% MeSo, 5% and 10% MeOH WCPA	10, 30, and 120	Sal. 28 ppt, temp. 20°C, <sup>a</sup> light 200 μmol photons m <sup>−2</sup> s <sup>−1</sup>

All treatments were composed of three repetitions

<sup>a</sup> Increased light intensity

experiment light intensity was increased, from 40 to 200 μmol photons m<sup>−2</sup> s<sup>−1</sup>.

All experiments had the following treatments with three repetitions: control—where cultures were not submitted to any freezing procedure; WCPA—without cryoprotectant; 5% and 10% dimethyl sulfoxide (Me<sub>2</sub>SO) and 5% and 10% methanol (MeOH) (Table 1).

Viability assessment and growth curve parameters

Viability was determined by the ability of cells to actively divide. After thawing, cultures were remained at rest to prevent loss of viable cells and to not compromise them. As soon as was possible to see a coloring in the cultures, as cells started to multiply exponentially, chlorophyll *a* (microgram per liter) was estimated by fluorescence analysis (Welschmeyer 1994). Samples of 10 mL were taken along the cultivation in order to obtain fluorescence values in vivo using a Turner TD-700 fluorometer. These values were then converted to levels of chlorophyll *a* by correlation curves previously determined. Correlation curves for each species were:

- *N. oculata*:  $y=1.8247x-39.829$   
 $R^2=0.9871$
- *T. weissflogii*:  $y=2.2338x+2.4255$   
 $R^2=0.9929$
- *Skeletonema* sp.:  $y=1.4538x-0.9484$   
 $R^2=0.9799$

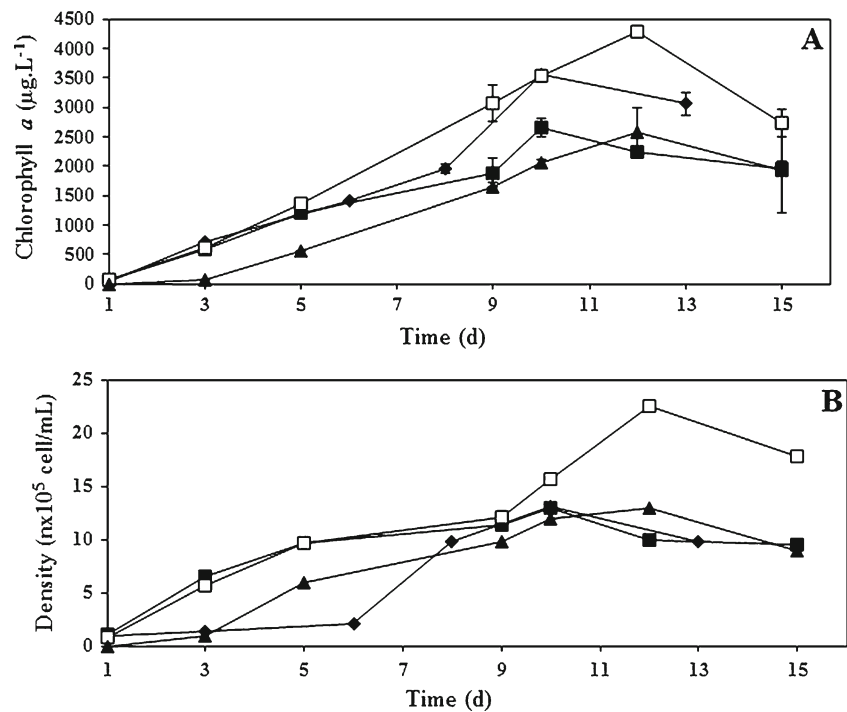
where *x* is the fluorescence value in vivo and *y* is the chlorophyll *a* level.

Cell density was also quantified by microscope counting using a Sedgewick-Rafter chamber (Sournia 1978) in the first and fourth experiment.

Statistics

The LAG phase, growth rate, and yield for chlorophyll *a* curves obtained in all experiments were compared statistically. Linear regressions were used to compare the growth rates of different treatments in the experiments. To verify the hypothesis that the regression lines were not all equal, an analysis of covariance to compare the *k* regression coefficients (*b*) (Zar 1999) was done. After the analysis of covariance the Tukey test was applied for multiple comparison of the regression coefficients (*b*) (Zar 1999). The data were not transformed for the linear regression because they met the assumption of linearity and normality of residuals (Kolmogorov–Smirnov test). The nonparametric Kruskal–Wallis test was applied to compare the maximum yield reached by the cultures in the different experiments. Statistical significance in all analysis was established at 0.05.

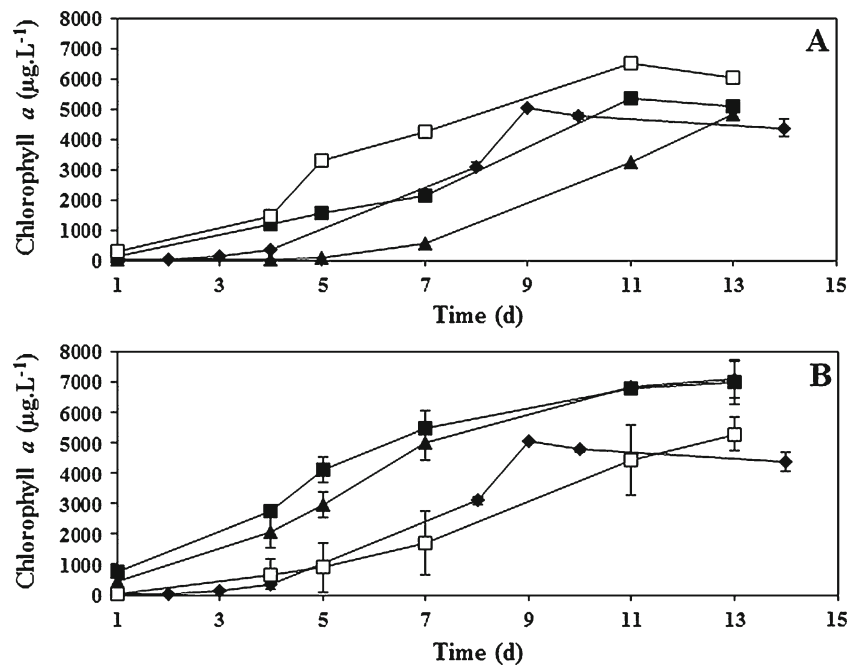
**Fig. 1** Experiment 1. Time course of thawed *Nannochloropsis oculata* cultures submitted to the rapid cooling test. After CPA treatment, cells were directly plunged into liquid nitrogen. **a** Chlorophyll *a* values. **b** Culture density. (Filled diamond control, filled square without CPA, empty square 5% dimethyl sulfoxide and filled triangle 10% dimethyl sulfoxide). Mean values  $\pm$ SD ( $n=3$ )



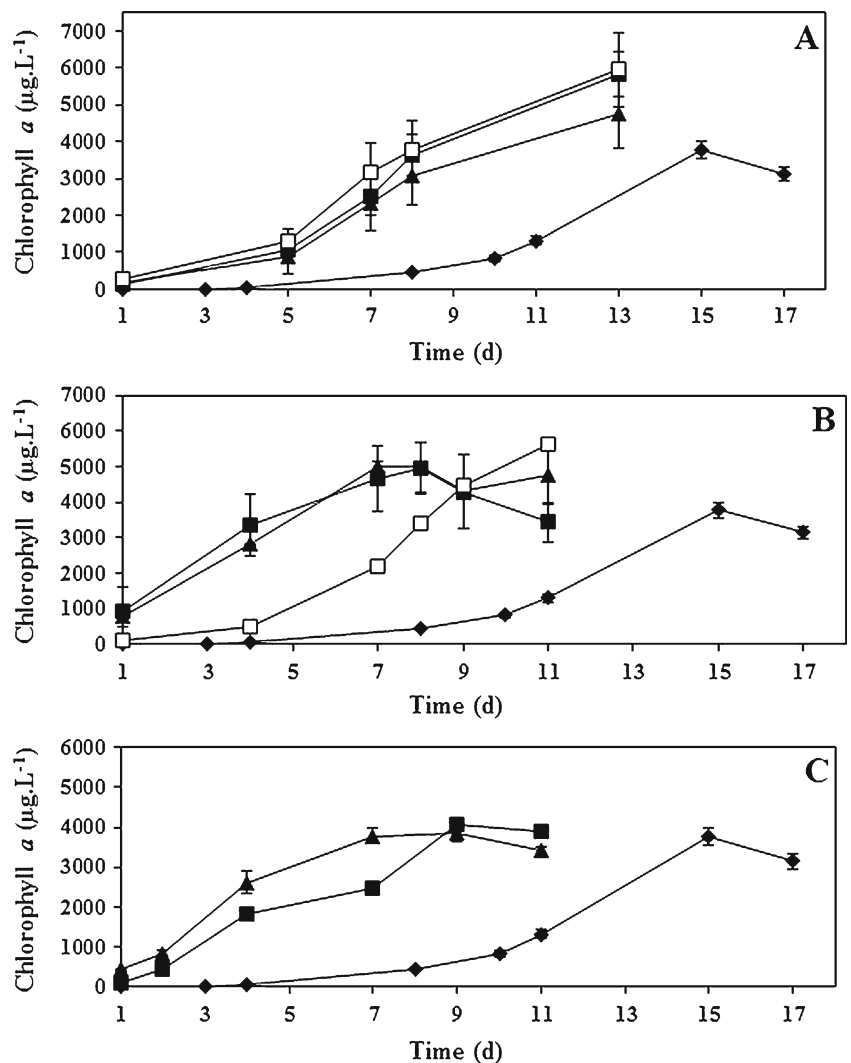
**Table 2** Growth curve parameters (chlorophyll *a*) for all experiments. LAG—length of LAG phase (days); growth rate ( $\beta$ ) ( $\mu\text{g L}^{-1} \text{day}^{-1}$ ); maximum yield. Different letters indicate significant statistical differences

Experiment	Species	Treatments	LAG	Growth rate ( $\beta$ ) ( $\mu\text{g L}^{-1} \text{day}^{-1}$ )	Maximum Yield ( $\pm$ SD)
1	<i>N. oculata</i>	Control	0	383.70 <sup>ab</sup>	3,548.38 ( $\pm$ 74.5) <sup>a</sup>
		Without CPA (WCPA)	0	262.81 <sup>b</sup>	2,657.53 ( $\pm$ 281.92) <sup>a</sup>
		5% DMSO	0	413.63 <sup>a</sup>	4,289.27 ( $\pm$ 9.0) <sup>a</sup>
		10% DMSO	2	282.27 <sup>b</sup>	2,565.39 ( $\pm$ 754.1) <sup>a</sup>
2	<i>N. oculata</i>	Control	1	656.59 <sup>a</sup>	4,764.61 ( $\pm$ 175.9) <sup>a</sup>
		WCPA	0	518.90 <sup>a</sup>	5,366.76 ( $\pm$ 136.8) <sup>a</sup>
		10% DMSO	4	611.53 <sup>a</sup>	4,841.24 ( $\pm$ 972.8) <sup>a</sup>
		5% MeOH	0	643.19 <sup>a</sup>	6,519.97 ( $\pm$ 990.9) <sup>a</sup>
3	<i>N. oculata</i>	Control	1	656.59 <sup>a</sup>	4,764.61 ( $\pm$ 175.9) <sup>a</sup>
		WCPA	0	519.99 <sup>a</sup>	6,969.45 ( $\pm$ 1348.3) <sup>a</sup>
		10% DMSO	0	585.14 <sup>a</sup>	7,060.69 ( $\pm$ 1068.8) <sup>a</sup>
		5% MeOH	0	469.52 <sup>a</sup>	5,271.26 ( $\pm$ 972.7) <sup>a</sup>
4	<i>N. oculata</i>	Control	2	280.77 <sup>b</sup>	3,767.71 ( $\pm$ 113.9) <sup>a</sup>
		WCPA (10 days)	0	498.52 <sup>a</sup>	5,806.21 ( $\pm$ 1108.5) <sup>a</sup>
		10% DMSO (10 days)	0	404.42 <sup>ab</sup>	4,773.73 ( $\pm$ 1771.2) <sup>a</sup>
		5% MeOH (10 days)	0	495.67 <sup>a</sup>	5,955.22 ( $\pm$ 1846.7) <sup>a</sup>
		WCPA (30 days)	0	458.18 <sup>ab</sup>	4,942.51 ( $\pm$ 126.6) <sup>a</sup>
		10% DMSO (30 days)	0	635.82 <sup>a</sup>	4,979.01 ( $\pm$ 432.2) <sup>a</sup>
		5% MeOH (30 days)	0	583.17 <sup>a</sup>	5,632.25 ( $\pm$ 326.4) <sup>a</sup>
		WCPA (120 days)	0	469.04 <sup>ab</sup>	4,075.78 ( $\pm$ 325.1) <sup>a</sup>
4	<i>T. weissflogii</i>	Control	0	169.69 <sup>b</sup>	2,404.36 ( $\pm$ 870.0) <sup>a</sup>
		10% DMSO (10 days)	0	169.48 <sup>b</sup>	1,644.04 ( $\pm$ 259.9) <sup>a</sup>
		5% MeOH (10 days)	0	325.52 <sup>a</sup>	2,929.26 ( $\pm$ 1360.7) <sup>a</sup>
		10% DMSO (30 days)	0	—	1,246.43 ( $\pm$ 230.5) <sup>a</sup>
		5% MeOH (30 days)	0	—	1,656.78 ( $\pm$ 245.7) <sup>a</sup>

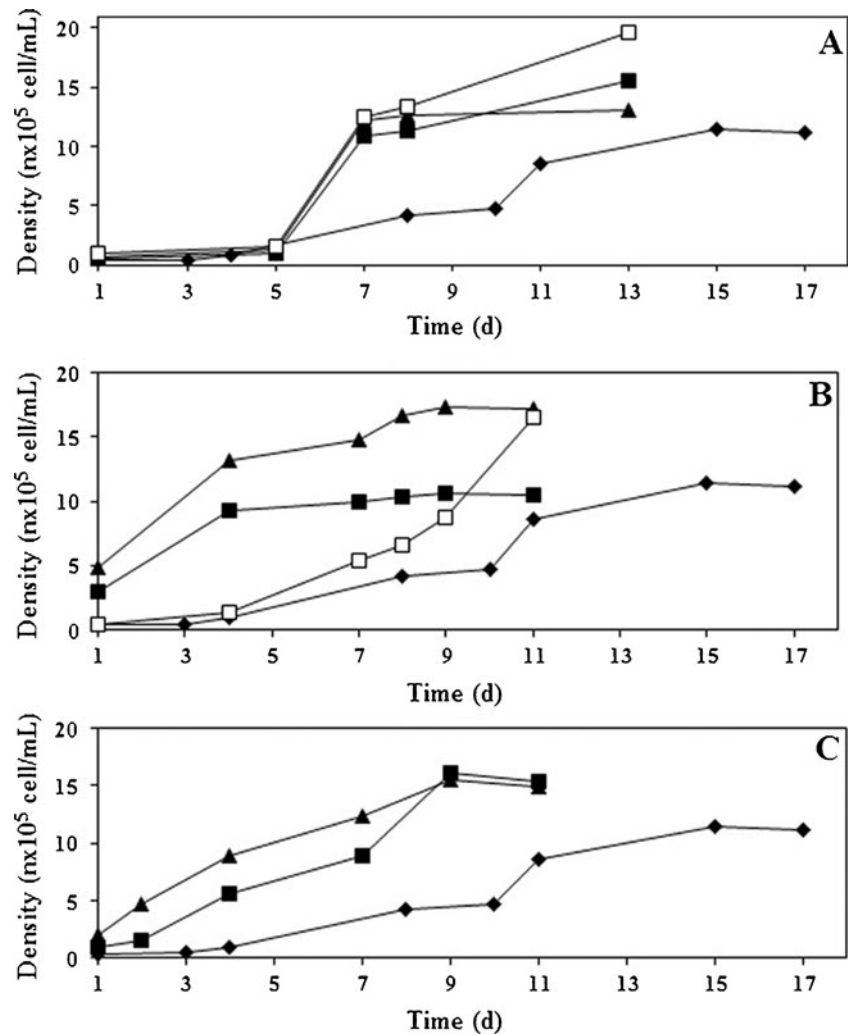
**Fig. 2** Chlorophyll *a* levels of thawed *Nannochloropsis oculata* cultures. **a** Experiment 2—Samples were placed into “Mr. Frosty” and kept at  $-18^{\circ}\text{C}$  for 24 h and then transferred to liquid nitrogen. **b** Experiment 3—Samples were introduced into “Mr. Frosty” and held at  $-80^{\circ}\text{C}$  for 1.5 h before being maintained in liquid  $\text{N}_2$ . (Filled diamond control, filled square without CPA, filled triangle 10% dimethyl sulfoxide, and empty square 5% methanol). Mean values $\pm$ SD ( $n=3$ )



**Fig. 3** Experiment 4. Chlorophyll *a* values of thawed *Nannochloropsis oculata* cultures submitted to the two-step controlled cooling protocol ( $-80^{\circ}\text{C}$  and liquid  $\text{N}$ ). **a** Samples stored for 10 days in liquid nitrogen and **b** samples stored for 30 days in liquid nitrogen. **c** Samples stored for 120 days in liquid nitrogen. All samples were cultured with  $200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ . Light intensity after thawing. Mean values $\pm$ SD ( $n=3$ ) (filled diamond control, filled square without CPA, filled triangle 10% dimethyl sulfoxide, and empty square 5% methanol)



**Fig. 4** Experiment 4—densities of thawed *Nannochloropsis oculata* cultures submitted to the two-step controlled cooling protocol ( $-80^{\circ}\text{C}$  and liquid N). **a** Samples with storage period in liquid nitrogen of 10 days. **b** Samples maintained in liquid  $\text{N}_2$  for 30 days. **c** Samples stored for 120 days in liquid nitrogen. All samples were cultured with  $200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ . Light intensity after thawing. Mean values  $\pm$ SD ( $n=3$ ). (Filled diamond control, filled square without CPA, filled triangle 10% dimethyl sulfoxide, and filled square 5% methanol)



## Results

### First experiment—rapid cooling protocol

Only *N. oculata* was successfully cryopreserved using the rapid cooling protocol. However, cell in the MeOH treatments did not survive the post freeze/thaw process. The treatments *N. oculata* without CPA (WCPA), with 5 and 10% dimethyl sulfoxide (5% Me<sub>2</sub>SO) displayed different chlorophyll *a* values for thawed cultures. Me<sub>2</sub>SO (5%) exceeded the control value on the 12th day ( $4,289.3\ \mu\text{g L}^{-1}$ ), whereas 10% Me<sub>2</sub>SO and WCPA stayed with the lowest values, reaching only 2,565.4 and 2,657.5  $\mu\text{g L}^{-1}$ , respectively (Fig. 1a; Table 2). Cell counting values presented similar trends as the chlorophyll *a* levels, with 5% Me<sub>2</sub>SO reaching the highest density ( $22.5 \times 10^5\ \text{cell mL}^{-1}$ ), after 12 days of growing (Fig. 1b). The 5% DMSO treatment showed the highest growth rate and yield, while cell in the 10% DMSO showed a poor performance with higher LAG phase and smaller growth rates than in the control (Table 2).

### Second experiment—two-step controlled cooling protocol in a freezer $-18^{\circ}\text{C}$

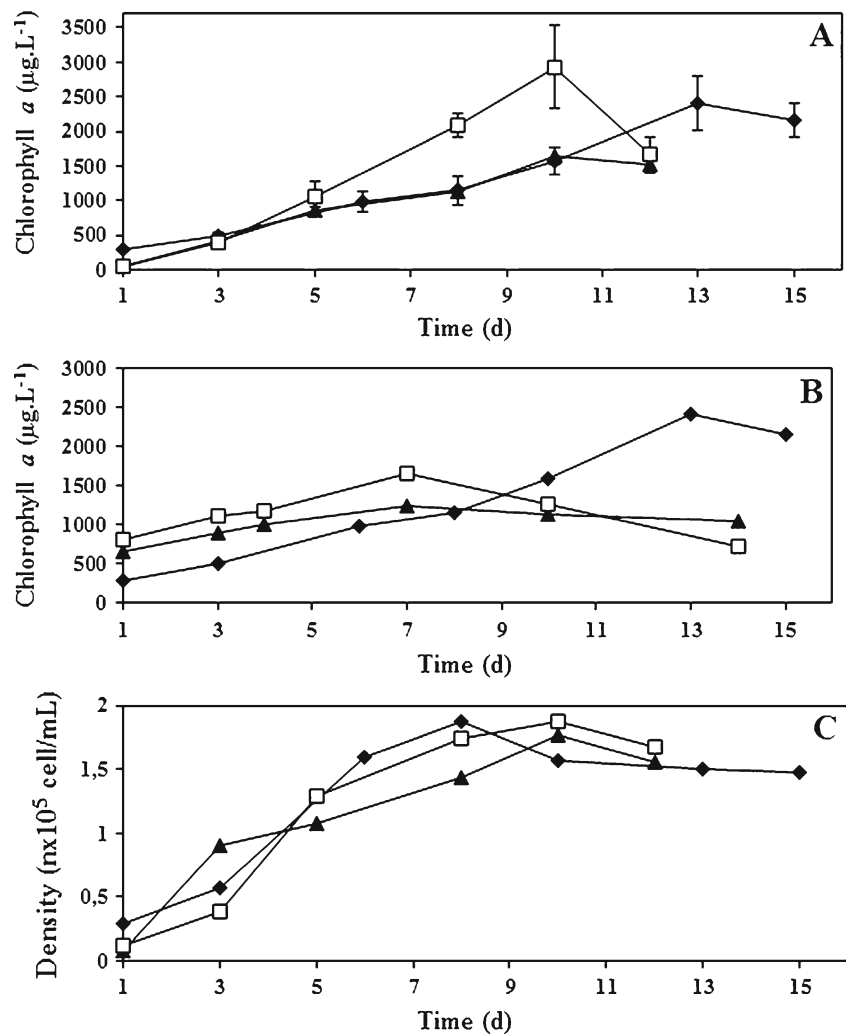
The smaller temperature ( $-18^{\circ}\text{C}$ ) in the first step of the cooling procedure proved to be suitable only for *N. oculata*, since *T. weissflogii* and *Skeletonema* sp. did not survive to any of the established treatments.

For *N. oculata*, yield values of the WCPA and 5% MeOH treatments surpassed the control, whereas 10% Me<sub>2</sub>SO demonstrated lower values and a delay in the initiation of the exponential growth phase (Fig. 2a; Table 2).

### Third experiment—two-step controlled cooling protocol in a freezer $-80^{\circ}\text{C}$

Similarly to the second experiment, of the three species tested in the third experiment, only *N. oculata* showed any post-thaw growth. Viability was observed with 10% dimethyl sulfoxide (10% Me<sub>2</sub>SO), 5% methanol (5% MeOH), and without CPA (WCPA). Chlorophyll *a* levels in 10% Me<sub>2</sub>SO

**Fig. 5** Time course of thawed *Thalassiosira weissflogii* cultures submitted to the two-step controlled cooling protocol. **a** Samples stored for 10 days in liquid nitrogen and **b** samples stored for 30 days in liquid nitrogen. **c** Cells abundance for samples stored for 10 days in liquid nitrogen. All samples were cultured with  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Light intensity after thawing. Mean values  $\pm$ SD ( $n=3$ ). (Filled diamond control, filled triangle 10% dimethyl sulfoxide, and empty square 5% methanol)



and WCPA treatments exceeded the control and did not present any LAG phase. On the other hand, 5% MeOH displayed a slower growth with its maximum in  $5,271.1 \mu\text{g L}^{-1}$  (Fig. 2b; Table 2).

Fourth experiment—different storage periods

*N. oculata* samples with storage period in liquid N<sub>2</sub> of 10 and 30 days were successfully cryopreserved with 10% dimethyl sulfoxide (10% Me<sub>2</sub>SO), 5% methanol (5% MeOH), and without CPA (WCPA) (Fig. 3a, b; Table 2). These treatments showed a shorter LAG phase and their chlorophyll *a* values exceeded the control. Highest chlorophyll *a* levels for samples of *N. oculata* cryo-stored for 10 days were measured for 5% MeOH and WCPA  $5,955.22$  and  $5,806.21 \mu\text{g L}^{-1}$ , respectively; Smaller values were obtained in the 10% Me<sub>2</sub>SO ( $4,773.73 \mu\text{g L}^{-1}$ ), though bigger than control (Fig. 3a; Table 2). In contrast, for samples melted after 30 days, highest values were obtained mainly in the 5% MeOH treatment, though showing an early declining phase

near the 7th day, similar to the treatments of 10% Me<sub>2</sub>SO and WCPA (Fig. 3b; Table 2).

Samples of *N. oculata* stored for 120 days in liquid nitrogen have demonstrated growth only with 10% Me<sub>2</sub>SO and without CPA. In both treatments, highest chlorophyll *a* values were close to the control. It was not possible to observe any post-thaw growth in the treatments prepared with MeOH as a CPA (Fig. 3c; Table 2).

For cultures of *N. oculata* cooled during 10 days, highest cell density was observed to 5% MeOH ( $19.6 \times 10^5 \text{ cells mL}^{-1}$ ). Me<sub>2</sub>SO (10%) and WCPA also exceeded the control, with  $13 \times 10^5$  and  $15.5 \times 10^5 \text{ cells mL}^{-1}$ , respectively (Fig. 4a).

Me<sub>2</sub>SO (10%) showed the maximum density near to  $15 \times 10^5 \text{ cells mL}^{-1}$  for cultures thawed after 30 and 120 days (Fig. 5b, c). The treatment WCPA did not demonstrate high abundances of cells after 30 days in cold ( $10.6 \times 10^5 \text{ cells mL}^{-1}$ ) (Fig. 4b), although it showed the highest density for samples cooled during 120 days ( $16.1 \times 10^5 \text{ cells mL}^{-1}$ ) (Fig. 4c). On the other hand, 5% MeOH showed density close to  $16.5 \times 10^5 \text{ cells mL}^{-1}$  when thawed after 30 days (Fig. 4b) but showed no significant growth

after 120 days (Fig. 4c). It was possible to observe post-thaw viability to *T. weissflogii* after a storage period of 10 and 30 days with 10% Me<sub>2</sub>SO and 5% MeOH (Fig. 5a, b; Table 2). For the cultures preserved for 10 days, 5% MeOH was the only one that surpassed the control. Both of the viable treatments melted after 30 days demonstrated chlorophyll *a* levels lower than the control at the end of the study period (Fig. 5b). No viability was achieved when samples of *T. weissflogii* were stored in liquid N<sub>2</sub> for 120 days.

*T. weissflogii* counts from samples melted after 10 days did not show significant differences between 10% Me<sub>2</sub>SO and 5% MeOH and control (Fig. 5c). The maximum density for 5% MeOH and the control was the same,  $1.87 \times 10^5$  cells mL<sup>-1</sup>. This parameter for 10% Me<sub>2</sub>SO was  $1.77 \times 10^5$  cells mL<sup>-1</sup>.

*T. weissflogii* did not resist freezing with no CPA treatment. On the other hand, *Skeletonema* sp. did not survive cryopreservation in all the treatments and tested storage periods.

## Discussion

Cryopreservation of microalgae presents many problems that hamper a broader use of this technique. When microalgae cells are subjected to freezing, low temperature storage, and subsequent thawing, they experience several chemical and physical stresses, which may result in damages and loss of cell viability (Taylor and Fletcher 1999).

Cell damage during freezing processes mainly occur due to large ice crystals formation but also due to osmotic pressure generated by salt imbalance. The high concentration of electrolytes and other solutes in frozen media produces a hypertonic condition that can lead to cell death (Pitt 1990). Therefore, the rate of cooling is extremely important since it determines the period of time in which microalgae are exposed to adverse conditions. Slow cooling rates tend to form external ice crystals before intracellular ice nucleation. When the temperature of extra-cellular water goes down, osmotic potential changes induce water to drive out of the cell. The extra-cellular increase in solute concentration may be lethal due to the higher water losses. Moreover, the cell deformation that occurs due to dehydration probably compromises normal microalgae metabolic activities after thawing. In theory, faster cooling rates are better as they mean shorter exposures to concentrated solutions (Canãvate and Lubian 1995a). On the other hand, slow cooling rates allow cells to remain turgid without much change in their solute concentration. However, intracellular ice formation occurs, though osmotic disequilibrium is reduced, also leading to cell death.

Cryoprotective agents (CPA) generally are added to offer protection from cell damage during freezing and thawing processes. CPAs with low molecular weight that passively

trespass the plasma membrane, as methanol and dimethyl sulfoxide, equilibrate the solute concentration between the cell interior and extra-cellular solution and have been utilized quite extensively for algal cryopreservation (Day and Brand 2005). These CPAs may act in different ways as lowering the temperature at which intracellular water freezes (Franks 1985), minimizing osmotically driven decrease in cell volume during freezing (Canãvate and Lubian 1995a) or altering membrane properties such as solute permeability (Santarius 1996). As one of important mode of action of cryoprotectants is the stabilization of membranes, and considering that membrane damage can be a primary initiator of free radical reactions, it is not surprising to find that several cryoprotectants may also act as free radical scavengers (Benson 1990).

CPAs and cooling protocols employed in this study showed different results among the three tested species. *Skeletonema* sp., for example, did not survive cryopreservation in any of the tested conditions. However, it is possible that the failure to preserve *Skeletonema* sp. in this work is related to the previous growth conditions of this strain. The *Skeletonema* sp. strain used in our study was obtained from the Phytoplankton and Aquatic Microorganisms Laboratory microalgae collection. The cells had low growth rates before and during the experiments. Problems derived from loss of quality stock cultures are very common when microalgae strains are kept for long in culture conditions (Canãvate and Lubian 1995b).

In contrast, results obtained with *T. weissflogii*, also a diatom, showed that this species could be preserved at ultra-low temperature (-196°C). Viability was obtained in samples cooled for 10 and 30 days with 10% dimethyl sulfoxide and 5% methanol. However, cultures thawed after 120 days did not survive. Consistent information about the cryopreservation of this species was not found in the literature, although Meyer (1985) reached viability in the cryopreservation of *T. weissflogii* with a longer adaptation time at -60°C in a two-step protocol. The production of extracellular polysaccharides by *T. weissflogii* (Borges et al. 2007) may confer some protection during freezing, giving to this diatom a greater chance of success than *Skeletonema* sp.

*N. oculata* was the most successful microalgae tested in this study, showing high tolerance to a wide range of cryopreservation conditions, similar to the one observed by Gwo et al. (2005). The species presented good responses when treated with 5% Me<sub>2</sub>SO, 10% Me<sub>2</sub>SO, 5% MeOH, and also without any CPA.

If effective protocols are established, long-term stability and viability can be ensured (Day 2004), so some of the difficulties found to cryopreserve microalgae can be attributed to protocols in current use. Most of them are capable of preserving a relatively limited range of algae, being largely restricted to small and simple shape species (Day 1998; Morris



1978). It is known that large size flagellate cells are recalcitrant to cryopreservation (Lourenço 2006) and require more refined protocols. Thus, it is likely that cell characteristics possibly influenced the results observed in this study since the three tested species are structural, morphologically, and phylogenetically different.

The eustigmatophyte *N. oculata* is a small green alga characterized by spherical or slightly ovoid cells, about 2–4 µm in diameter and robust cell wall (Chrétiennot-Dinet et al. 1993). *Skeletonema* sp., in the class Bacillariophyceae, is a centric chain forming diatom, characterized by cylindrical cells, from 5 to 16 µm in diameter, linked together by silica tubes to form long chains of variable length (Bérard-Therriault et al. 1999). *T. weissflogii* is also a centric diatom, with plain and circular valves, about 9–18 µm in diameter (Fryxell and Hasle 1977).

We have observed that depending on CPAs concentration, microalgae performance was not the same. *T. weissflogii* survived cryopreservation with similar growth in 10% Me<sub>2</sub>SO and 5% MeOH. The growth curve parameters of thawed cultures calculated for *T. weissflogii* are near to the control ones; however, 5% MeOH showed a higher yield. MeOH or Me<sub>2</sub>SO concentrations smaller than 2% (v/v) are seldom effective, while concentrations higher than 12% (v/v) are toxic (Day and Brand 2005). Therefore, concentrations of 5% and 10% (v/v) were employed, as also suggested for the freezing of other microalgae species (Tzovenis et al. 2004).

The small eustigmatophyte, *N. oculata*, was successfully cryopreserved with 5% and 10% Me<sub>2</sub>SO and 5% MeOH. Similarly, other studies have shown that a large range of cryoprotective solutions can afford significant cryoprotection to *N. oculata* (Poncet and Véron 2003). However, the experiments carried out with *N. oculata* showed that methanol was the most effective CPA for a slower cooling rate (second experiment), with the 5% MeOH treatment showing the highest growth. In contrast, when faster cooling rates were employed, Me<sub>2</sub>SO produced the best results. It was observed in the fourth experiment that 10% Me<sub>2</sub>SO displayed similar amounts of viability independently of the time that cells were kept in freezing, whereas 5% MeOH showed a capricious performance. With 5% MeOH as a CPA, samples stored for 10 and 30 days showed a high growth, but after 120 days of storage, they did not survive. It will be inappropriate to establish the most effective CPA for *N. oculata* since in the treatment without CPA, the cells also survived and achieved high viability levels. These results suggest that the addition of CPAs do not necessary mean any advantages for cryopreservation of these algal cells. However, the fact that two concentrations of Me<sub>2</sub>SO (5% and 10%) were effective and only one of MeOH (5%) may indicate that Me<sub>2</sub>SO better protects *N. oculata* cells or that MeOH is more toxic for this species.

In order to estimate potential natural freezing tolerance, a treatment was prepared without any CPA addition. Among

the species employed, only *N. oculata* was successfully cryopreserved in this condition. Poncet and Véron (2003) reported that *N. oculata* have the absolute need for an appropriate cryoprotective solution to prevent cells from lethal cryoinjuries. This necessity was observed in this study for *T. weissflogii*, of which samples without CPA did not re-grow. However, this consideration is sufficiently different of that found in this study for *N. oculata*, where samples of this species without CPA demonstrated viability in all experiments with growth equal or higher than the control in all cryostorage periods. Moreover, other species of this genus also showed excellent viability after freezing without the use of any cryoprotectant (Canãvate and Lubian 1995a). It is likely that certain cell characteristics contributed to the excellent performance of *N. oculata* in freezing conditions. Its resistance to cryoinjuries could be related to cell membrane permeability and structural conditions of cell wall found in most species of this genus. Similarly, the mucilage produced by these cells may act as a natural cryoprotective agent.

Measurements of re-growth for *N. oculata* showed that cell densities in algal cells after cryopreservation increased faster than those of control cells. This increase may be related to the reproduction strategy of *N. oculata* involving autospores, which are non-flagellate spores that have the same morphology as the parent cell (Hoek et al. 1995). The stress caused by cryopreservation process may stimulate a reproduction by autospores, resulting in a fast and significant increase of cell number when thawed cultures begin to growth.

Other possibility for the high growth rates presented by *N. oculata* after thawing might be mixotrophy of some residues that would favor a faster and bigger growth of the cells after cryo-storage. However, both hypothesis (autospores and mixotrophy) remain to be tested.

In this study, the different cooling rates led to important differences in each employed protocols. When freezing was carried out by directly plunging the samples into liquid nitrogen, only *N. oculata* survived. Probably, the structure of cell wall attenuated negative effects of osmotic disequilibrium during the ice nucleation conferring some resistance to this species.

During the second experiment (using a –18°C pre-treatment), melted samples presented a longer LAG phase, whereas in the third experiment (with a –80°C pre-treatment) thawed cultures started the exponential growth phase 3 to 5 days after being inoculated in fresh *f/2* medium. Latter recovery of cells in the modified –18°C freezer protocol may be attributed to (1) the increased time of exposure to cryoprotectants, (2) excessive dehydration, and/or (3) toxicity of high extra-cellular solute concentrations in the unfrozen component of the medium. With a faster cooling, however, as in the protocol with a –80°C freezer, cooling process was optimized and damages associated with ice formation were minimized.

Borges et al. (2007) demonstrated by *P* vs *I* curves that *T. weissflogii* and *Skeletonema* sp. had higher productivity when

light intensity was near to  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , so it was supposed that an increase in light level might excite culture growth. Since *T. weissflogii* and *Skeletonema* sp. did not survive in the third experiment, light intensity was changed in the fourth experiment from 40 to  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . However, the alteration in light intensity resulted in an evident growth change only for *T. weissflogii*'s samples, which showed viability only in the fourth experiment. This fact shows that, prior to establishing cryopreservation protocols, culture conditions and manipulations are as important as freezing and thawing procedures (Day et al. 2000).

In summary, this study has shown that *N. oculata* can be successfully cryopreserved even without CPA, while *Skeletonema* sp. did not survive in low temperatures. *T. weissflogii*, on the other hand, has a complex set of interacting variables that must be taken into consideration when attempting the long-term freezing preservation of this species (Steponkus et al. 1992). Thus, we may conclude that cryopreservation has considerable potential as microalgae conservation technique as observed for these and other algal strains. However, the difficulty in establishing common protocols for different microalgae species was clear from this study. All these facts indicate that the use of empirical approach has its limitations and the development of improved methodologies requires a better understanding of cell damage during freezing and thawing conditions.

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