Effect of two freezing methods on growth and physiological response in the cryopreservation of the microalgae *Chaetoceros muelleri* and *Phaeodactylum tricornutum* and the cyanobacterium *Synechococcus elongatus*

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INTRODUCTION

Microalgae and cyanobacteria are photosynthetic organisms of great ecological importance, with high potential in aquaculture feed, biofuel production, drug manufacturing, and pigment extraction. However, maintaining strain collections is costly, time-consuming, resource-intensive, and carries the risk of crop loss. In this regard, cryopreservation ensures the availability of strains, preserves their genetic integrity, and significantly reduces maintenance costs. This study evaluated the effect of cryopreservation on growth, cell size, and physiological response in *C. muelleri*, *P. tricornutum*, and *S. elongatus*, using controlled and uncontrolled freezing methods.

METHODS

Cryoprotectant incubation

The microalgae were incubated with 10 % DMSO for 10 min at 22 + 1°C and aspirated in 0.5-mL French straws.

Freezing methods

Two freezing methods were evaluated using three devices:

Controlled cooling rate: -3 °C/min using a **cryochamber**.

Uncontrolled cooling rate: -8 °C/min using a **polystyrene box** or **Cryo Rack**.

Storage

Samples were stored for 1, 7, and 14 days to evaluate growth, and for 6 months to evaluate the physiological response.

Thawing

Thawing was performed in a seawater bath at 45°C for 11 s.

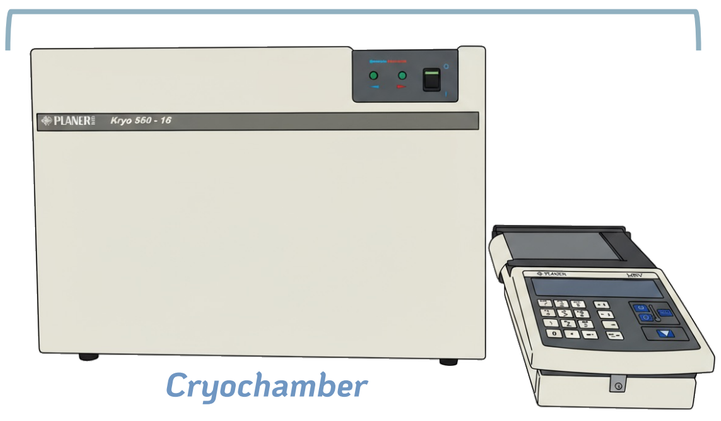
Growth evaluation

Cell growth was determined by measuring optical density (O.D.) for 10 days.
An ANCOVA was performed to compare the freezing methods.

Physiological response evaluation

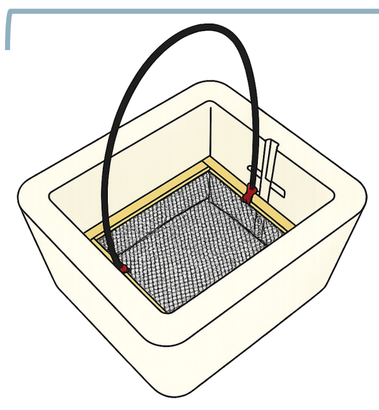
Best freezing method was selected based on the cell growth analysis to evaluate reactive oxygen species (ROS), viability, apoptosis, and cell proliferation by flow cytometry after 6 months of storage.

Controlled freezing

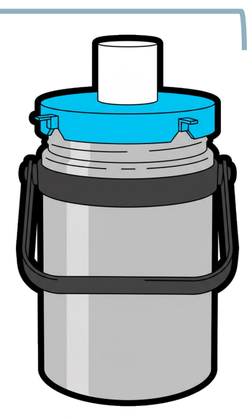


Cryochamber

Uncontrolled freezing



Polystyrene box



Cryo Rack

RESULTS

No significant differences ($p > 0.05$) were found in the growth of the 3 species frozen in any of the freezing methods (**Figure 1**).

The duration of the adaptation phase was similar between freezing methods, but different among species (**Figure 1**).
P. tricornutum showed the fastest recovery (3 days), followed by *C. muelleri* (5 days), while *S. elongatus* exhibited the longest adaptation phase (8 days).

The ROS analysis showed that all species experienced oxidative stress after thawing (time 0 h) (**Figure 2**). However, *C. muelleri* and *P. tricornutum* exhibited a reduction of more than 90 % in ROS levels after 24 h. In contrast, *S. elongatus* showed a lower recovery capacity compared with the other two microalgae species.

Flow cytometry analysis allowed the distinction of viable cells, cells undergoing apoptosis, and cells with loss of membrane integrity (**Figure 3**). *S. elongatus* and *P. tricornutum* maintained viabilities above 70 %, whereas *C. muelleri* showed values below 25 %.

The proliferation analysis from 0 to 24 h post-thawing (**Table 1**) showed that all three species exhibited high percentages of divided cells. *P. tricornutum* reached 100 % divided cells, followed by *C. muelleri* and *S. elongatus*, both exceeding 95%.

Table 1. Post-cryopreservation cell proliferation of two microalgae species and a cyanobacterium. Uncontrolled freezing (polystyrene box).

Species	Sample	Time	Proliferation Index	Percentage of Divided Cells (%)
C. muelleri	Experimental control	0 h	3.97	43.5
		24 h	4.39	97.2
	Treatment	0 h	1.81	71.6
		24 h	2.33	95.3
P. tricornutum	Experimental control	0 h	1.73	66.8
		24 h	2.59	100.0
	Treatment	0 h	1.99	79.5
		24 h	3.03	100.0
S. elongatus	Experimental control	0 h	1.56	53.9
		24 h	3.23	89.2
	Treatment	0 h	2.15	72.1
		24 h	3.40	97.3

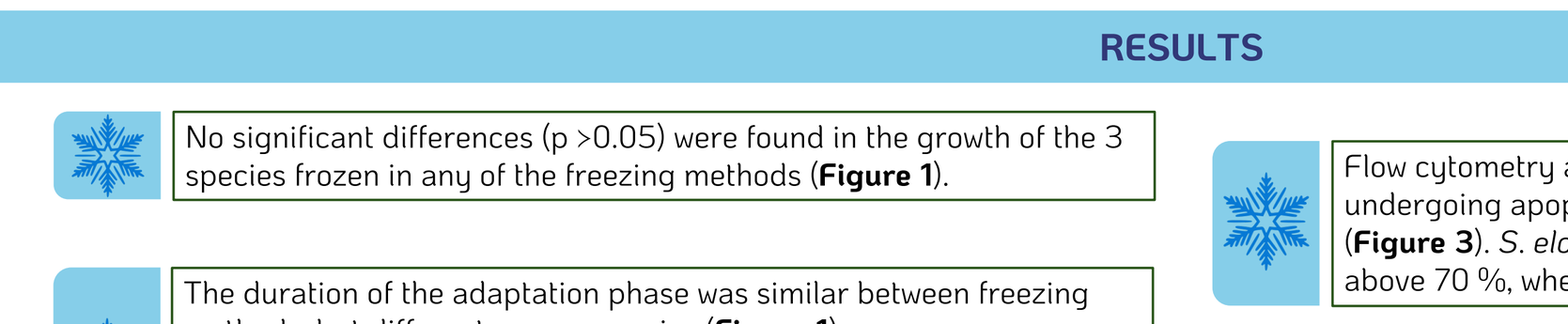


Fig. 1 Cell density (Log_2) after 14 days of storage. **A**, *C. muelleri*; **B**. *P. tricornutum*, and **C**, *S. elongatus*. Green line, experimental control; purpure line, cryochamber; orange line, polystyrene box, and blue line, Cryo Rack.

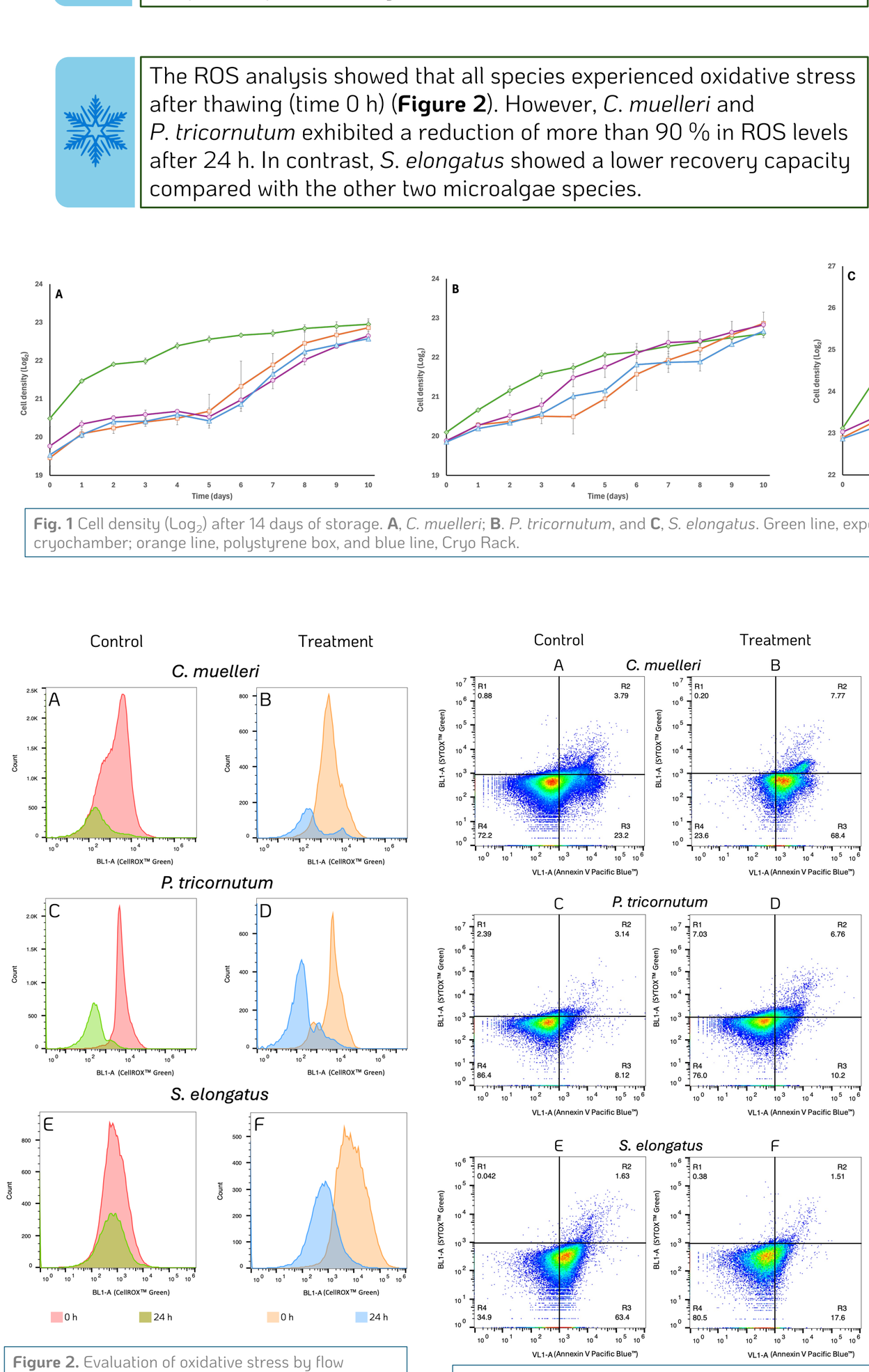


Figure 2. Evaluation of oxidative stress by flow cytometry in two microalgae species and a cyanobacterium post-cryopreservation with and without DMSO. Fluorescence was measured immediately after thawing (0 h) and after 24 h of recovery.

Figure 3. Evaluation of viability and apoptosis by flow cytometry in two microalgae species and a cyanobacterium post-cryopreservation with and without DMSO. R1: non-viable; R2: late apoptosis; R3: early apoptosis; R4: viable.

CONCLUSIONS

The post-cryopreservation growth evaluation demonstrates that both freezing methods are equally effective after 14 days of storage in all three evaluated species.

Although growth recovery was successful in all species, differences were observed in the duration of the adaptation phase, possibly associated with structural and physiological characteristics specific to each species.

The three species showed the ability to recover from post-thaw oxidative stress; however, the magnitude of this response depended on the antioxidant mechanisms specific to each species.

Post-cryopreservation viability showed a species-specific effect of DMSO: favorable in *S. elongatus* and *P. tricornutum*, but reduced in *C. muelleri*, suggesting cytotoxicity or osmotic damage in this diatom.

The elevated levels of cell proliferation during the first 24 h post-thawing indicate that the three species maintained physiological functionality and recovery capacity after cryopreservation.

Acknowledgments

We thank to SECIHTI for the scholarship awarded. This work was supported by CICESE funds (project no. 623142). We thank to L. Trujillo for technical suppor.