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# Cryopreservation of the green microalga *Tetraselmis suecica* with a controlled, slow-cooling technique

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## Abstract

Cryopreservation is a common technique for long-term storage of living cells and tissues. Herein we investigated the possibility of cryopreserving the green microalga *Tetraselmis suecica*, which is used in animal nutrition and biotechnological applications. Cryopreservation was carried out with a controlled rate freezer with one of three concentrations (1, 2, or 3 M) of each of five cryoprotectants: methanol, dimethyl sulfoxide (DMSO), glycerol, propylene glycol, and ethylene glycol (each for 30, 45, or 60 min). Post- freezing vitality was evaluated by both cellular ATP content and growth, and samples incubated in 1 M DMSO for 60 min (pre-cooled at 8°C/min) demonstrated higher ATP content and growth rates. These findings will aid others interested in cryopreserving this model alga.

**Key words:** Cryopreservation, Algae, Controlled slow cooling, Culture, Cryoprotectant

## Introduction

In recent years, global interest in microalgae has increased exponentially (Pienkos and Darzins, 2009; Di Genio et al., 2021), and their vast biotechnological potential has pushed several companies to invest in mass-producing them for the food, pharmaceutical, renewable energy, and other technological industries (Larkum et al., 2012; Brasil et al., 2017; Santana et al., 2017; Brasil et al., 2017; Kapoore et al.,

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2019; Paredes and Bellas, 2019). Traditionally, they are cultured in batch under controlled conditions (continuously or semi-continuously) (Day and McLellan, 1995), with some having been cultured for decades (Day et al., 1997). However, long-term culture is expensive and can result in genetic (or epigenetic) modifications (Jaworski et al., 1988; Day and DeVille, 1995; Day et al., 2000), spillage, contamination, or other sources of human error. As an alternative, algae can instead be cryopreserved at ultra-low temperatures (<-130°C) (Lin and Tsai, 2020; Tsai and Lin, 2012) to maintain their vitality post-thawing (Day and Brand, 2005). Cryopreserved algal cultures do not experience genetic changes or contamination, and maintenance costs are cheaper (Brand and Diller, 2004; Chong et al., 2016a). However, algal cryopreservation is difficult, and protocols vary widely across species; progress has consequently been slow (Abreu et al., 2012; Chong et al., 2016b).

Cooling rate optimization is critical for successful cryopreservation since the speed with which water is cooled plays a fundamental role in the possibility of formation of intracellular ice crystals that arise from incomplete dehydration and can result in membrane destruction and cell death. Although crystal formation is promoted at rapid cooling rates (Tsai et al., 2015), slow cooling rates elicit osmotic stress and, more generally, increase the probability of cryoprotectant (CPA) toxicity (Lin et al., 2019a, b). Despite these difficulties, microalgae such as *Chlorella vulgaris*, *Isochrysis galbana*, *Dunaliella salina* have been successfully cryopreserved using two-step freezing techniques (Guermazi et al., 2010), as well as other protocols (Taylor and Fletcher, 1998; Rhodes et al., 2006). That said, most algal species lack cryopreservation protocols. For instance, no protocol exists for the green microalga *Tetraselmis suecica*. We therefore sought herein to use a two-step freezing technique (achieved by a programmable freezer) to optimize the cryopreservation of this species.

## Materials and methods

**Cell culture.** *T. suecica* (20 mL) from the Laboratory of Algae Physiology of the Università Politecnica delle Marche (Italy) was diluted 5-fold in 100 mL flasks containing media prepared as follows: 32 g of Salt Water Mix (Coralife, Franklin, USA) added to 1 L of deionized water) and then filtered through a 45-µm filter (Thermo-Fisher Scientific [TFS], USA). After autoclaving, 30 mL of 50x F/2 media (Sigma, Germany) were added to 970 mL of this solution under a laminar flow hood. The microalgal cultures were kept in the

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culture chamber (TFS) with fluorescent lamps at 25°C; the photoperiod was 12/12 hr light/dark (150-190 μmol $m^{-2}$ $s^{-1}$). Prior to experiments, cells were transferred to 50 mL tubes, and densities were assessed from 1-mL aliquots that were first fixed in Lugol's solution (Lugol, 1829) and then counted within Neubauer chamber (Marienfeld, Germany) under a microscope. Only cultures with densities of 1.5-2 x $10^6$ cells/mL were used in experiments outlined below.

**Cryopreservation and response variables.** Cells were pelleted at 2000 *xg* for 3 min and resuspended in one of the following CPAs after decanting the supernatant: 1, 2, or 3 M of dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), propylene glycol (PG; J.T. Baker, USA), glycerol (GLY; J.T. Baker), ethylene glycol (EG; J.T. Baker), or methanol (MeOH; Merck, Germany). After incubating for 15, 30, 45, or 60 min at 25°C, the samples were loaded into 0.25 μL plastic micro-straws (IMV Technologies, France), inserted into straw holders, and placed in a CryoMed™ controlled-rate freezer (TFS). The temperature was dropped from 25 to -6°C at 3°C/min and held for 10 min. Then, the temperature was brought to -40°C at either 2, 4, or 8°C/ min. Next, the temperature was kept at -40°C for 10 min before immersion in liquid nitrogen for 2 hr. Finally, the straws were thawed in a water bath (Major Science, Taiwan) at 37°C for 10 s, and the samples were immediately coated onto glass tubes to measure ATP content using the ApoSensor™ ATP cell viability bioluminescence assay (Milpitas, USA) and a Lumat 9507 luminometer (Berthold Technologies, Germany). The manufacturer's protocol was used except only half of the luciferase enzyme volume recommended was used (from 10 to 5 μL). Cell densities were estimated as described above, and 1 mL of cells was diluted to 25 mL in F/2 media. Every week, 10% of the media was exchanged, and cell densities quantified after another 28 and 56 culture days.

## Results

Cells cultured after cryopreservation with 1 and 2 M DMSO possessed the highest ATP content at a cooling rate of 8°C/ min (Fig. 1a). A concentration of 1 M was also best for PG (Fig. 1b), though overall low ATP levels were documented for PG-cryopreserved cells. GLY concentrations of 1 and 2 M yielded cells with higher ATP content than controls (Fig. 1c) at 8°C /min, with 1 M being better (as also observed for EG; Fig. 1d). Of all treatments, cells incubated with 1 M MeOH for 60 min were characterized by the highest ATP levels (Fig. 1e). The result of the present study demonstrated that slow cooling rate of 2 and 4ºC /min gave significantly lower ATP content than a cooling rate of 8ºC /min (Fig. 2 and Fig. 3).

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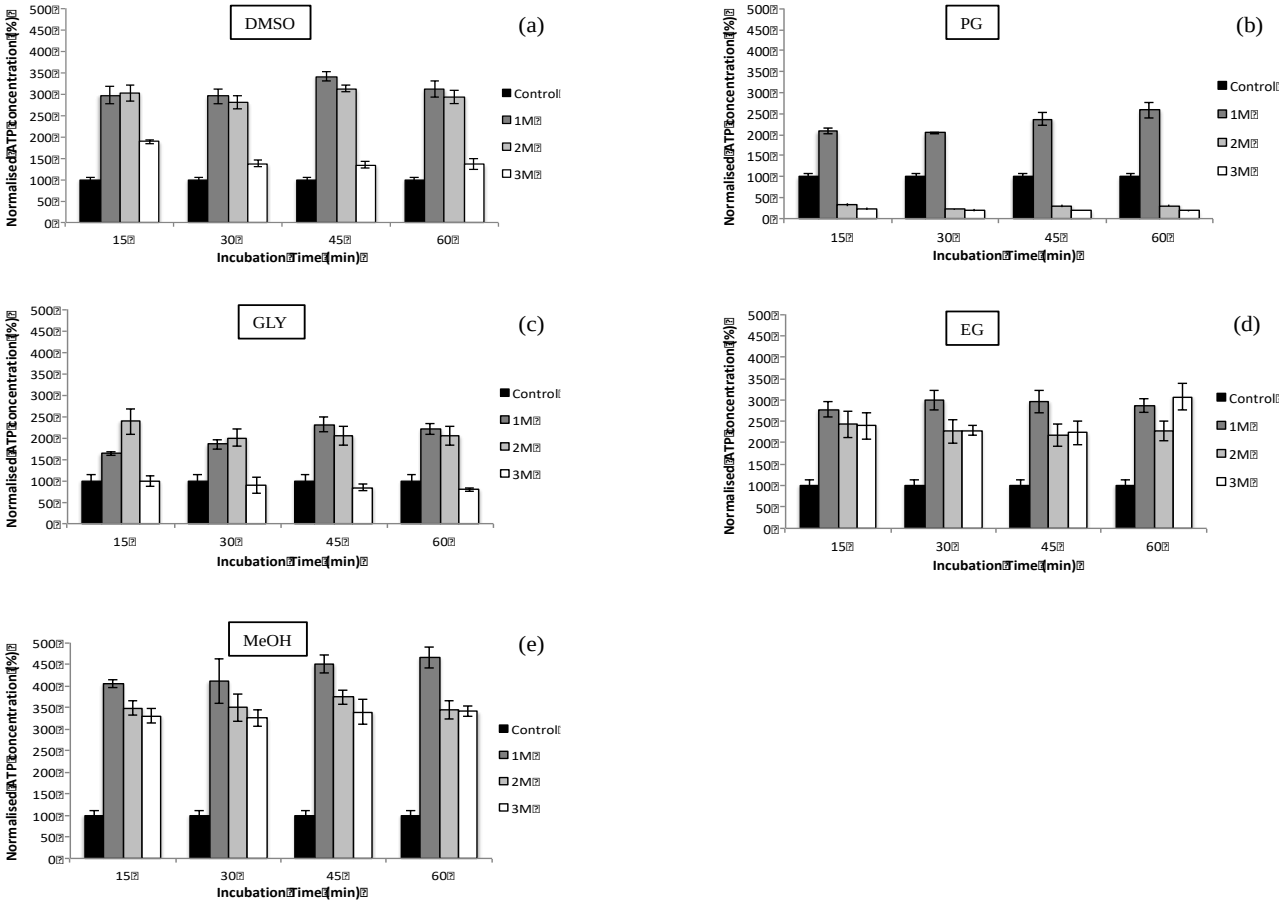


**Fig. 1.** ATP content of *T. suecica* cells cryopreserved at 8°C/min with several CPAs (tested at different concentrations and incubation times). Values have been presented as relative to the control (set at 100%) and error bars have been represented the standard deviation of the mean. The experiment was repeated three times.

Similarly, post-thaw cell growth was best after cryopreservation with DMSO (Fig. 4a) and MeOH (Fig. 4e), with the latte being superior. Cells cryopreserved with the other three CPAs failed to grow appreciably (Fig. 4b-d).

## Discussion

*T. suecica* was successfully cryopreserved, and, in line with previous studies, two-step freezing did not appreciably compromise viability (Taylor and Fletcher, 1998; Morris, 1978; Kuwano et al., 1993). From the ATP assay data, MeOH appeared to be the optimal CPA, in agreement with Tanaka et al. (Tanaka et al., 2001). Furthermore, it has been shown that this alcohol also has a protective action on the lipid membranes, which are otherwise compromised by freezing stress (Yang and

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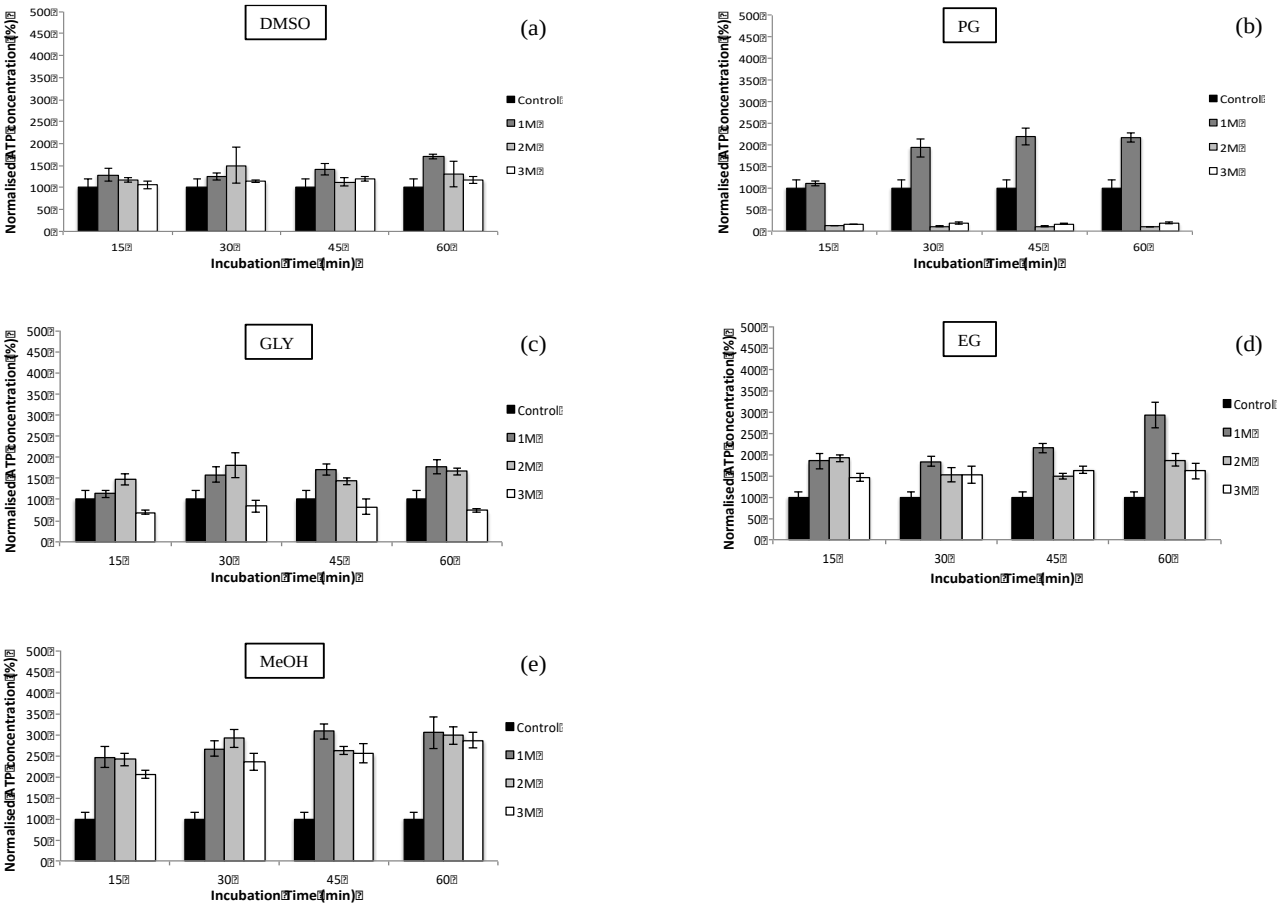


**Fig. 2.** ATP content of *T. suecica* cells cryopreserved at 4°C/min with several CPAs (tested at different concentrations and incubation times). Values have been presented as relative to the control (set at 100%) and error bars have been represented the standard deviation of the mean. The experiment was repeated three times.

Li, 2016). However, even for MeOH, post-thaw growth rates never reached those of the controls. This could be due to impartial elimination of the CPAs from the cells; residual CPAs could have consequently induced toxicity (Fahy, 1986; Arakawa et al., 1990). This is especially likely given that Tanaka et al. (Tanaka et al., 2001) found that methanol was three-fold more permeable than other CPAs, notably DMSO (which gave better results in terms of cell growth rates herein). Others have also found that DMSO causes a less significant effect than methanol on cell growth rates post-cryopreservation (Cañavate and Lubian, 1994; Guermazi et al., 2010).

*T. suecica* has also been successfully

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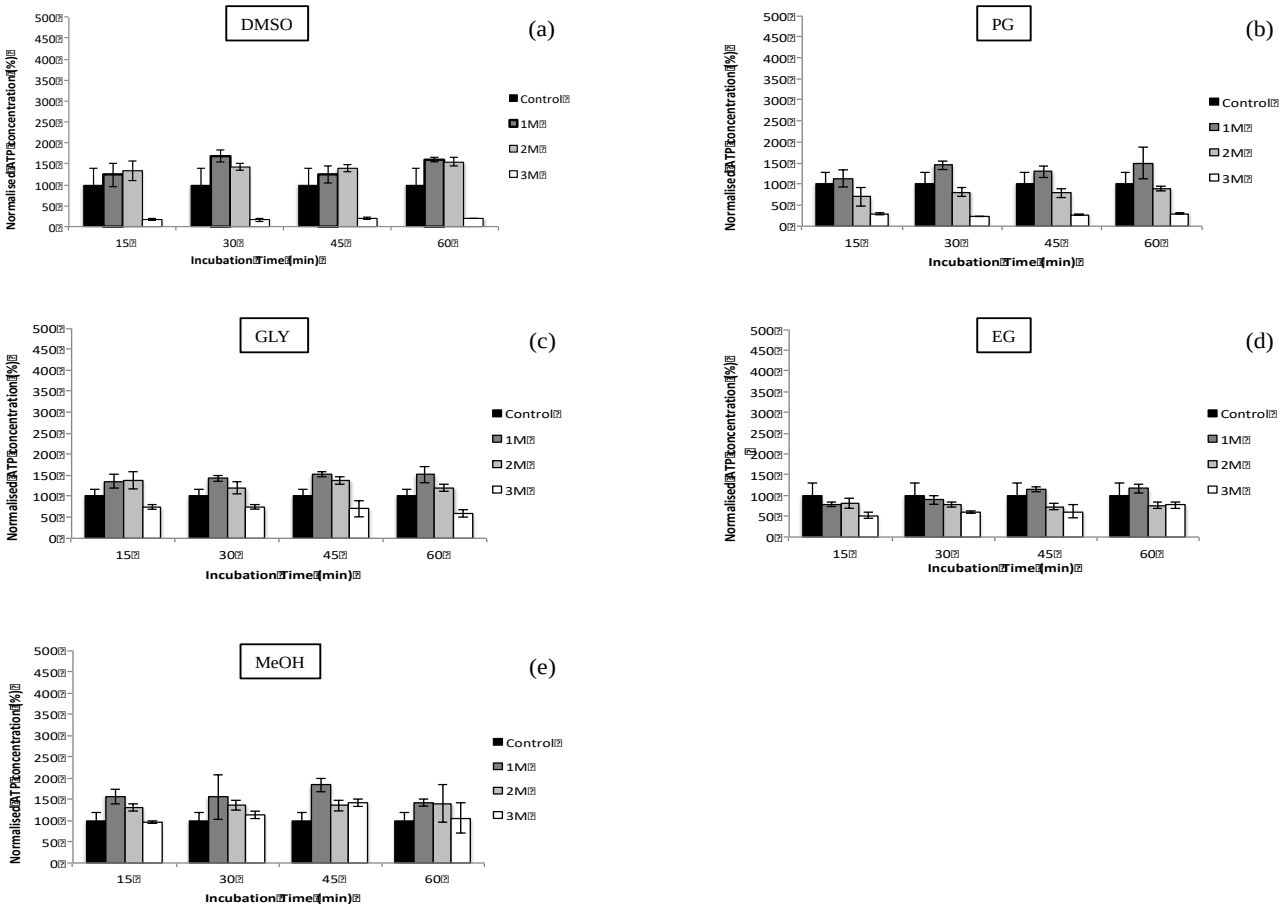


**Fig. 3.** ATP content of *T. suecica* cells cryopreserved at 2°C/min with several CPAs (tested at different concentrations and incubation times). Values have been presented as relative to the control (set at 100%) and error bars have been represented the standard deviation of the mean. The experiment was repeated three times.

cryopreserved with GLY (Fenwick and Day, 1992; Day and Fenwick, 1993), while GLY was toxic to *Nannochloropsis oceanica*, *N. salina*, and *Nannochloropsis sp*. (Youn and Hur, 2009). However, our data revealed GLY to be a poor CPA. In the end, these studies all used different freezing rates and so cannot be directly compared. It is possible that GLY's high viscosity could mean it requires more time to penetrate cells (Lovelock and Bishop, 1959; Kiyosawa, 1993), and others have indeed used far longer incubation times than ours (two hours) (Hubalek, 2003).

EG is normally used at concentrations of ~2-40% (v/v) for cryopreservation of yeast (Mazur, 1960), mushrooms (Sakurada et al., 1995), algae

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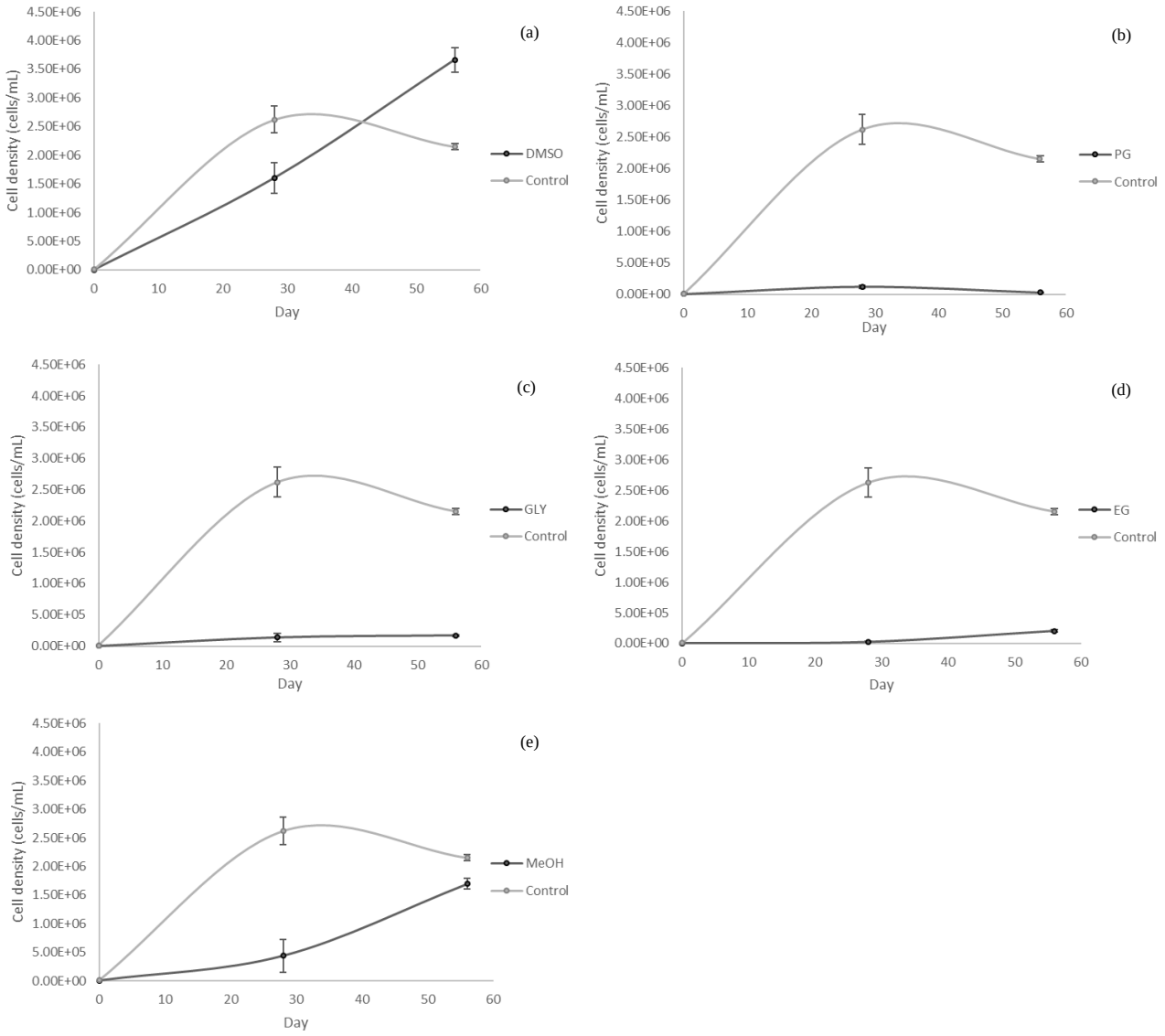


**Fig. 4.** Cell densities of cryopreserved cultures of *T. suecica* using a cooling rate of 8°C/min and 1 M of a) DMSO, b) PG, c) GLY, d) EG, or e) MeOH. Error bars represent the standard deviation of the mean. The experiment was repeated three times.

(Kono et al., 1997), protozoans (Simione Jr and Daggett, 1977), and invertebrates (Cirino et al., 2019; Tsai et al., 2010). Herein, samples cryopreserved with EG failed to grow. This could be because the associated diols could have partially dissolved the polysaccharides in the cell walls (Nash, 1966). Superior results were instead obtained with a 60-min incubation with 1 M DMSO, followed by freezing at 8°C

*Platax* 18: 07-16, 2021
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/min. However, ATP provides only a snapshot of cellular energy content; it is possible that some cells may be possess low ATP levels yet still be viable (Chong et al., 2016c). Future studies could attempt to resolve this, as well as determine the exact timing of infiltration of CPAs into cells; this will facilitate comprehending how/if they induce appreciable toxicity.

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