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Cryopreservation of *Pyramimonas mucifera*: Effect of DMSO and the slow cooling method on viability

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A Dissertation submitted to the Faculty of Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Master's in Science (Animal, Plant and Environmental Sciences).

Under the Supervision of Prof. David Mycock and Prof. Stuart Sym.

Johannesburg, 2021

DECLARATION

I, Karabo Thato Mokoena, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master's in Science (Animal, Plant and Environmental Sciences) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

X 

Karabo Mokoena

22 day of February 20 22 in Bloemfontein

PUBLICATION

Mokoena, K., Sym, S. and Mycock, D. (2022). Cryopreservation of *Pyramimonas mucifera*. *CryoLetters*, 43(1): 18-24.

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ABSTRACT

Microalgae are diverse and important for scientific research and applicability to industry. Microalgal cultures are expensive to maintain for such purposes and require frequent batch culturing which has adverse effects that could lead to the loss of algal diversity. To preserve this diversity, cryopreservation has been identified as an appropriate conservation measure. The aim of this study was to determine the effect of 10 % dimethyl sulfoxide (DMSO) and the two-step cooling method on cell viability and ultrastructure of *Pyramimonas mucifera* (Chlorophyta, Pyramimonadophyceae, Pyramimonadales, Pyramimonadaceae). Viability was inferred using membrane integrity and mitochondrial function. Fluorescent stains propidium iodide (PI) and Rhodamine 123 (Rh123) were used, respectively, to determine these aspects. The ultrastructural response to this protocol was characterised by focusing on the membrane integrity of the plasma membrane, chloroplast, and mitochondrion of *P. mucifera* cells. DMSO was proven to be an appropriate cryoprotectant as it caused minimal damage to cell viability (<30%) and ultrastructure, and cells were uncompromised upon its removal (>90% recovery). The two-step cooling method caused a decrease in cell viability, mechanical damage, and signs of apoptosis in some cells compared to untreated and DMSO treated cells. However, the method was found to be acceptable for this species as viable cells were always encountered and could re-establish into vigorous cultures after cryopreservation.

Keywords: Cryopreservation; DMSO; Propidium iodide; *Pyramimonas mucifera*; Rhodamine 123; Two-step cooling; Viability; Ultrastructure**.**

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LIST OF ABBREVIATIONS

CO_2	Carbon Dioxide
CPA	Cryoprotective Additives / Cryoprotectant
CTCF	Corrected Total Cell Fluorescence
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
FOV	Field of View
IBP	Ice Binding Protein
MF	Mitochondrial Function
O_SO_4	Osmium Tetroxide
PI	Propidium Iodide
RFU	Relative Fluorescent Unit/s
Rh123	Rhodamine 123
ROS	Reactive Oxygen Species
SSU rRNA	Small subunit ribosomal ribonucleic acid
UD	Undescribed Subgenera

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1. Introduction and Rationale

1.1. Background

Algae form a unique, but artificially classified group of organisms that are of scientific interest due to their diversity and applicability in various commercial sectors. These organisms are important as they form the foundation of many aquatic food chains (Guillard, 1975; Day & Fenwick, 1993; Hecky & Hesslein, 1995; Rhodes *et al.,* 2006) and in some countries, they are a staple food source for human consumption (Crawley *et al*., 2009). In industry, microalgae have also become important in the production of biodiesel and other renewable resources (Abreu *et al*., 2012). Furthermore, the presence of these species in marine environments has been linked to decreased levels of carbon dioxide (CO_2) in the atmosphere and thus are being used for biotechnology to mitigate climate change (Ono & Cuello, 2006; Farelly *et al*., 2013).

Species belonging to the genus *Pyramimonas* (Chlorophyta) provide an example of microalgae with an intrinsic scientific interest, due to their genetic diversity (Suda *et al.,* 201333 *Pyramimonas* is regarded as one of the most primitive genera of green algae, as this species has scales that cover their plasmalemma, rather than a cell wall (Figure 1.1) (McFadden *et al*., 1986, Daugbjerg *et al*., 1994, Graham *et al*., 2009). Members of this genus are free-living and flagellate, characteristically pyramidal to globular, with a cup-shaped chloroplast, and a large, single, reticulated mitochondrion (McFadden & Wetherbee, 1982) (Figures 1.1 & 1.2).

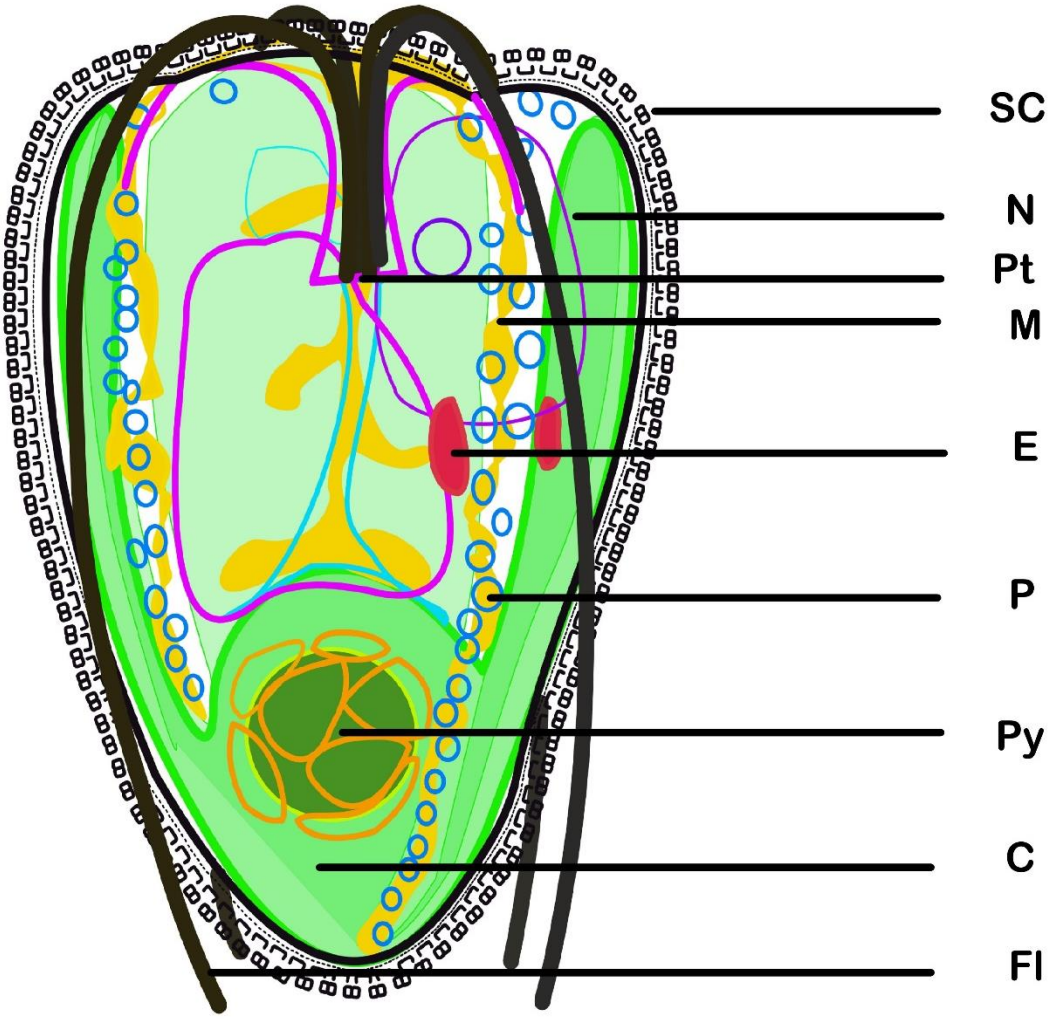


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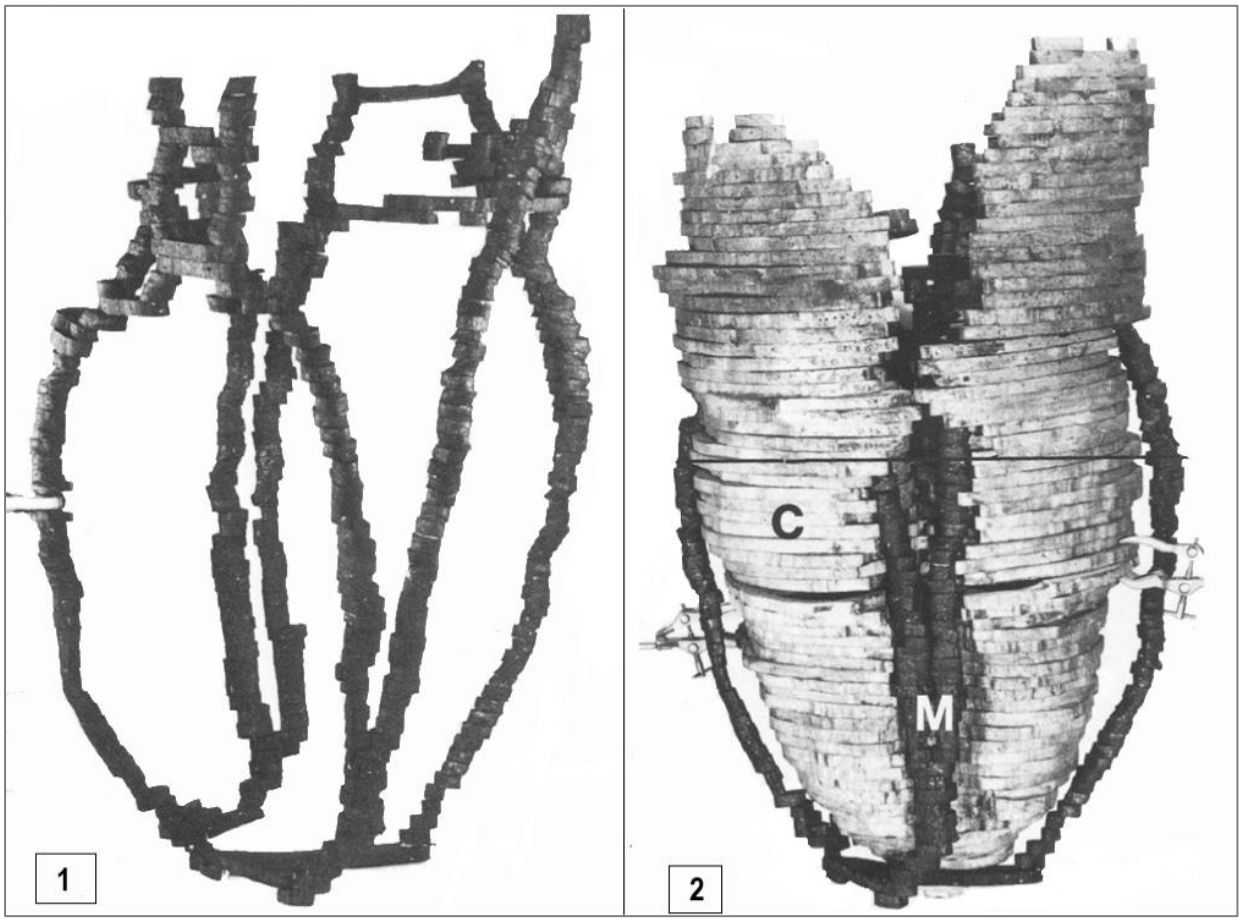


Figure 1.2: Shape of the mitochondrion in *Pyramimonas gelidicola*. (1) Structure of mitochondrion on its own. (2) The mitochondrion (M) is branched throughout the cell and closely associated to the chloroplast (C) (McFadden & Wetherbee, 1982).

Six subgenera have been described in the genus, but only four have clear phylogenetic support (Round 1971, Daugbjerg *et al*., 1994; Moro *et al*., 2002; Graham *et al*., 2009; Harðardottir *et al*., 2014). These four subgenera (*Vestigifera, Trichocystis, Pyramimonas* and *Punctatae*) are characterized according to scale morphology, internal ultrastructure, and their biochemical features (McFadden *et al*., 1986; McFadden *et al*., 1987), and these distinctions are reflected by phylogenetic inference from gene sequences (Fig. 1.3) (Daugbjerg *et al*., 1994; Viprey *et al*., 2008).

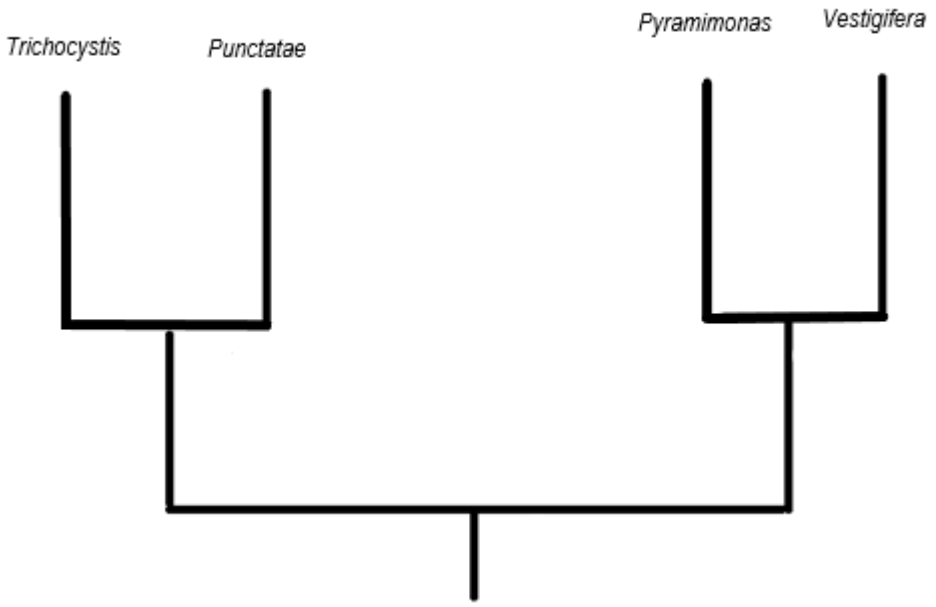


Figure 1.3: Phylogenetic tree of the subgenera of the genus *Pyramimonas*, inferred by the ML method from SSU rDNA sequencing (Viprey *et al*., 2008).

Pyramimonas mucifera Sym & Pienaar, a member of the subgenus *Punctatae*, is characterised as a bright yellow-green cell that is dorsi-ventrally flattened and weakly lobed (Fig. 1.4) (Sym & Pienaar, 1991). The cells of *P. mucifera* range from 11.9 – 22.1 µm in length and 9.35 – 16.2 µm in width and contain a flagellar pit (one-quarter of the cell length) from which four heterodynamic flagella exit (Sym & Pienaar, 1991). The cells have two longitudinal rows of puncta between the lobes that begin from the flagellar pit and extend towards the base of the cell (Fig. 1.4) (Sym & Pienaar, 1991). Cells of this species are filled with muciferous vesicles that distribute the subcellular organelles into "small pockets of cytoplasm" (Sym & Pienaar, 1991). In culture the cells of *P. mucifera* form both benthic (populating the bottom of the culture vessel) and planktonic (swimming actively in the water column) populations, but are predominantly benthic (Sym & Pienaar, 1991). Cells in the benthic population are surrounded by mucilage and oscillate gently moving the mucilage (Sym *et al*., 2000). Planktonic swimming is achieved by recurved flagellar beating in this species (Sym *et al*., 2000).

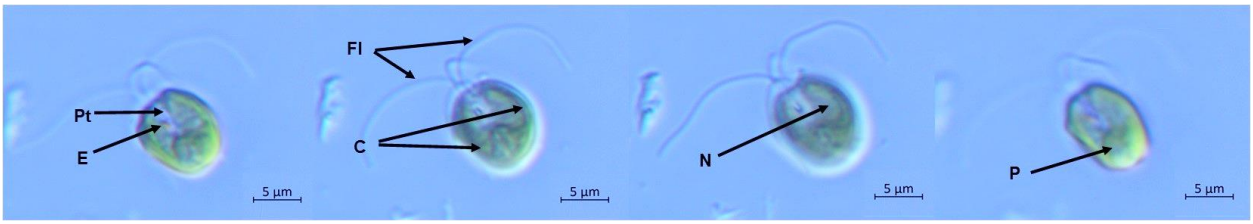


Figure 1.4: Through-focus micrograph of a cell of *Pyramimonas mucifera* (DIC optics). **Key:** Pt = flagella pit; E = eyespot; Fl: Flagella; C = chloroplast; N = nucleus; P = pyrenoid.

For scientific or biotechnological purposes, these cultures need to be established and maintained. Microalgal cultures are commonly maintained through batch culturing, which demands frequent sub-culturing because of their ability to rapidly accrue biomass over a short period (Lorenz *et al.*, 2005). This rapid accumulation of biomass results in a constant depletion of nutrients in the medium (Day *et al*., 2000; Cañavate & Fernandez-Diaz, 2001; Gwo *et al.*, 2005). Batch culturing of extensive culture collections requires space, expensive culture equipment, considerable effort for their effective maintenance, and runs the risk of losing algal diversity through contamination and/or genetic drift (Day *et al.*, 1997; Day *et al*., 2000; Gwo *et al.*, 2005). As a consequence, cryopreservation has been recommended as an appropriate long-term storage technique (Gwo *et al.*, 2005; Tanniou *et al*., 2012).

1.2. Literature Review

Cryopreservation is the technique by which cells, tissues and in some cases, organs are preserved at sub-zero temperatures and fixed into "a state of suspended animation" (Douzou, 1977; Saks, 1978; Karlsson & Toner, 1996). This allows the morphology, physiology, biochemistry, and genetic material to be fixed at cryogenic temperatures predominantly in liquid nitrogen (- 196 ℃) (Saks, 1978; Karlsson & Toner, 1996). It was pioneered by Polge *et al.* in the 1940s, with the vitrification and dehydration of spermatozoa. Cryopreservation has since been applied to different organisms (plants and animals) and is regarded as a good method for long term storage of materials (Karlsson & Toner, 1996; Day *et al*, 1997), due to the

organisms being preserved without alteration to their chemical, biological or physical processes (Karlsson & Toner, 1996).

1.2.1. Cryopreservation of Microalgae

Tolerance of green alga, *Nitella*, to a sub-zero temperature of – 20℃ (Cohn, 1871), introduced the use of cryopreservation as a conservation method in algae and has since been used as an alternative to batch culturing (Paredes *et al*., 2020). However, to date research is still limited to species that have economic importance and have biotechnological application (Rhodes *et al*., 2006; Abreu *et al*., 2012; Paredes *et al*., 2020). This interest skews the focus of conservation away from that covering the greater genetic diversity of algae. The first reports to be published on successful cryopreservation of microalgae occurred in the 1960s (Holm-Hansen, 1963; Leibo & Jones, 1963; Hwang & Horneland, 1965), with a few reports on the cryopreservation of macroalgae. Recently successful cryopreservation has been reported to be "predictable" based on cell morphology and phylogenetic origin (Fernandes *et al*., 2019). These can be used to inform researchers of the appropriate culture age, ideal cryoprotectant and its appropriate concentration for successful cryopreservation (Fernandes *et al*., 2019). A range of microalgae have been successfully cryopreserved using a variety of methods and cryoprotectants (Table 1). Successful cryopreservation methods for microalgae have incorporated both dimethyl sulfoxide (DMSO) (Fernandes *et al.*, 2019; Paredes *et al*., 2020) and the slow cooling method (Table 1).

Table 1: Microalgae species that have successfully been cryopreserved.

Alga	**Rate of Cooling**	**Cryoprotectant**	**Reference**
Unicellular Chlorophyceae	Uncontrolled freezing	None	Holm-Hansen, 1963
Chlamydomonas reinhardtii	Slow Cooling	10 % Glycerol, 5 % DMSO	McGrath & Daggett, 1977
Chlamydomonas reinhardtii	Slow Cooling	1 – 5 % DMSO	Gresshoff, 1977
Chlorella sp., *Tetraselmis suecica*,	Slow Cooling	5 - 20 % DMSO	Gilboa & Ben-Amotz, 1979
Tetraselmis suecica	Slow Cooling	5 - 15 % ethanol, glycerol & methanol	Fenwick & Day, 1992
Tetraselmis suecica, *Tetraselmis chui*	Slow Cooling	5 – 10 % DMSO & glycerol	Day & Fenwick, 1993
Unicellular Chlorophyceae	Slow Cooling	5 % methanol	Bodas *et al*., 1995
Chlamydomonas reinhardtii	Encapsulation-Dehydration	0.5 M sucrose	Hirata *et al*., 1996
Chlorella emersonii, *Tetraselmis suecica*	Slow Cooling	5 % DMSO 10 % glycerol & methanol	Day *et al*., 1997
Chlamydomonas reinhardtii	Slow Cooling	2 – 10 % methanol	Crutchfield *et al*., 1999

Alga	Rate of Cooling	Cryoprotectant	Reference
Collection of cyanobacteria and green algae	Rapid & Slow Cooling	5 – 10 % DMSO & methanol	Saadaoui *et al.*, 2016
Desmodesmus spinosus, *Chlorella sorokiniana*, *Chlamydomonas biconvexa*	Slow Cooling	10 % DMSO	Fernandes *et al.*, 2019
Diverse group of microalgae from different culture collections.	Slow Cooling	10 – 15 % DMSO	Paredes *et al*., 2020

1.2.2. Stress Response in Microalgae

Generally, biological systems do not respond well to environmental stresses - especially extreme temperature changes. A decrease in temperature to sub-zero levels causes substantial changes to the physical and chemical state of the intracellular and extracellular water, which affects the normal functioning of the cell (Fuller *et al*., 2004). At sub-zero temperatures extracellular water freezes which causes cell dehydration and ice crystal formation of the intracellular water (Karlsson & Toner, 1996; Fuller *et al*., 2004) and reduces biological metabolism (Gao & Critser, 2000). The concept of freezing for long-term preservation is based on the idea of inhibiting chemical and physiological processes in biological systems without cryo-injury (Taylor & Fletcher, 1999). In order to prevent cryo-injury, the cell's responses to the changes associated with freezing need to be understood and mitigated.

Cryo-injury – Biological causes

Generally, organism do not respond well to temperatures below their preferred threshold and as a result these low temperatures cause a decrease in membrane fluidity, swelling and disorganization of the chloroplast and mitochondria, and the enlargement of the Golgi vesicles and the endoplasmic reticulum (Kratsch & Wise, 2000; Nagao *et al*., 2008). Ultra-low temperatures that are used for cryopreservation could have more adverse implications and lead to cell death.

There are various modes of death and biological processes that occur when a cell is exposed to stresses, and these can cause molecular and morphological changes (Darzynkiewics *et al.*, 1997). Each mode of death varies in the way these changes occur in response to stress. Apoptosis and necrosis are the most widely understood modes of death. Apoptosis, sometimes referred to as programmed cell death, has been simply described as "cell suicide" (Darzynkiewics *et al.*, 1997). It is an active process in which a cell executes its own death through a series of processes in response to a stimulus (Figure 1.5) (Darzynkiewics *et al.*, 1997). Necrosis is the response of a cell to injury or cytotoxic agents and is considered as "accidental cell death" (Darzynkiewics *et al.*, 1997).

The apoptotic response of unicellular microalgae to stresses is like apoptosis in both plants and animals (Bidle & Falkowski, 2004; Affenzeller *et al*., 2009; Zuppini *et al.*, 2010). Apoptosis occurs biochemically and through changes to the cell's ultrastructure. Biochemically, there is an activation of caspase activity, degradation of DNA and expression of *de novo* proteins (Bidle & Falkowski, 2004). Morphologically, the cell shrinks; chromatin condensation occurs; plasmalemma membrane integrity is maintained; autophagosomes and vacuoles are present in the cytoplasm; and there is fragmentation of mitochondria (Lizard *et al*., 1995; Bidle & Falkowski, 2004; Youle & Karbowski, 2005; Arnoult, 2006; Affenzeller *et al*., 2009).

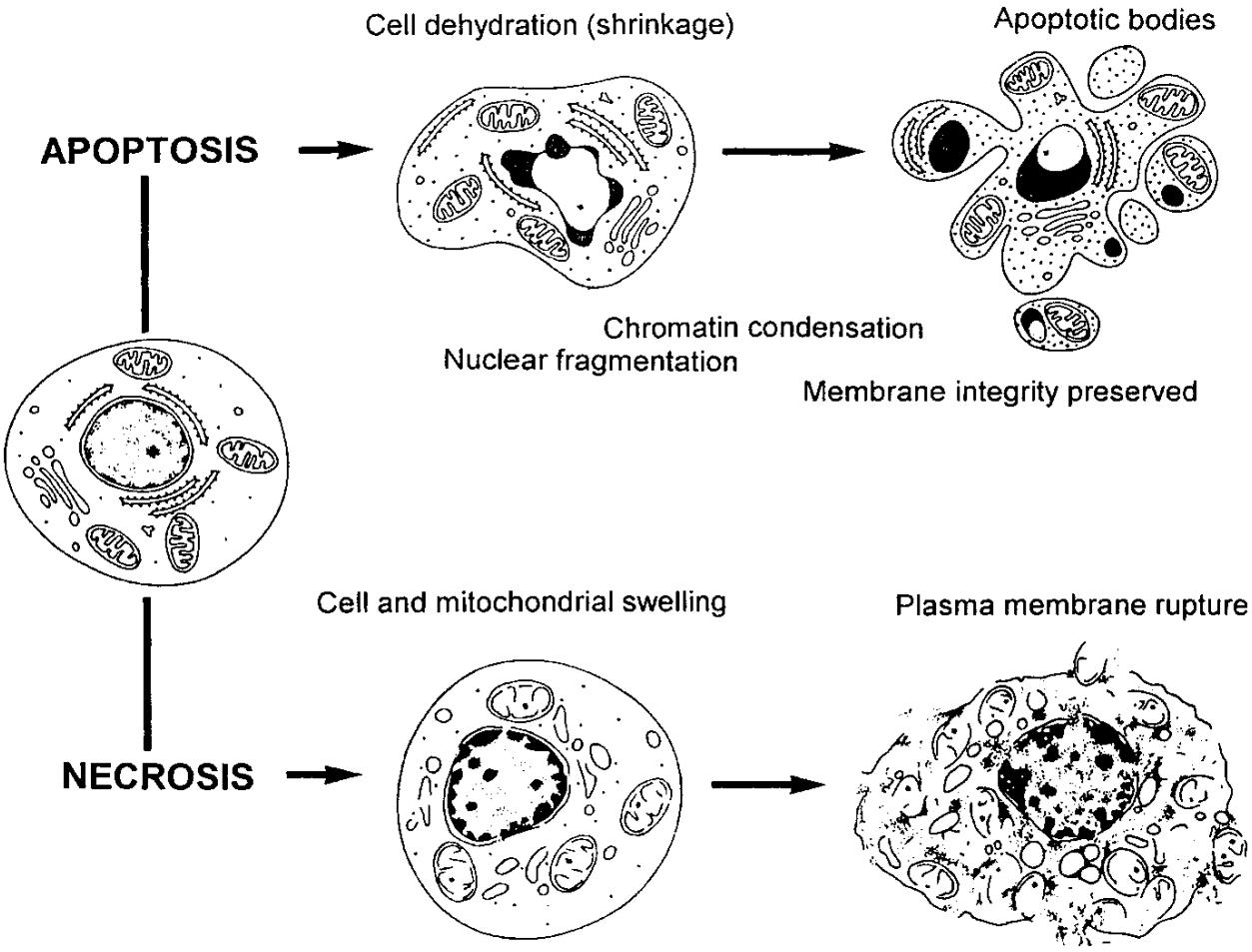


Figure 1.5: Morphological and biochemical changes that occur in the cell during the two described modes of death (from Darzynkiewics *et al*., 1997).

Biochemical response

Oxidative stress is a biochemical change that can cause cellular damage during cryopreservation procedures (Steffen & Palta, 1989). Oxidative stress is a result of the production and accumulation of reactive oxygen species (ROS) within the cell (Bremner & Benson, 2004; Reddy & Raghavendra, 2006; Bui *et al*., 2013). In photosynthetic species, photooxidative stress can occur when ROS are generated because an excessive amount of light is absorbed, which can cause an over-reduction of the electron transport chains (Reddy & Raghavendra, 2006). This leads to the accumulation of ROS which causes damage to lipids and proteins (Lesser, 2006; Martin *et al*., 2007). Damage to lipids (lipid peroxidation) can result

in changes to membrane fluidity and integrity (Lesser, 2006; Martin *et al*., 2007). Accumulated ROS can attack proteins resulting in alterations of amino acid conformation and specificity, in turn altering protein structure and function (Lesser, 2006; Martin *et al*., 2007). The cellular sites for ROS production are the chloroplast, mitochondria, endoplasmic reticulum and microbodies (Lesser, 2006). Mitochondria are the sites of the highest ROS production, as it is a by-product of mitochondrial respiration (Martin *et al.*, 2007). The cell has a range of mechanisms to prevent ROS overproduction, however if the cell is unable to prevent ROS accumulation this may trigger apoptosis (Martin *et al.*, 2007). In the mitochondria alone, oxidative stress may cause damage which can lead to the dysfunction of the respiratory system (Martin *et al.,* 2007). This will suppress mitochondrial metabolism through lipid peroxidation leading to loss of energy production and ultimately cell viability (Martin *et al*., 2007). Therefore, ROS play a role in triggering cell death. ROS are also produced in the chloroplast in a similar manner to the mitochondria and the presence of light during cryopreservation protocols can have an impact on the cryo-survival by triggering cell death. Consequently, the incorporation of light and dark cycles into cryo-protocols have been recommended to prevent photooxidative stress (i.e., overproduction or accumulation of ROS) by incubating the cells in the dark after treatment with DMSO and immediately post-thaw after DMSO dilution (Day & Brand, 2005).

Cryo-injury – Physical causes

Osmotic Stress

Osmotic stress has been identified as a major cause of cryo-injury during the cryopreservation procedure (Mazur, 1984; Jungare *et al*., 2021; Whaley *et al*., 2021). The mechanisms of cryopreservation involve movement of water from the intracellular matrix, to decrease the amount of water present in the cell to prevent ice crystal formation (Meryman, 1971a; Mazur,

1984; Muldrew *et al*., 2004). This movement of water causes a change in solute concentration around and within the cell (Gao & Crister, 2000). A change in solute concentration causes a change in the osmotic equilibrium (Karlsson & Toner, 1996; Gao & Crister, 2000). An osmotic equilibrium refers to the equilibration of water and solute concentration between the intracellular and extracellular matrix (Suescún-Bolívar & Thomé, 2015). This change in osmotic equilibrium can cause cell damage (Suescún-Bolívar & Thomé, 2015), hence the importance of maintaining this gradient during the freeze/thaw process.

Ice crystal formation

The most common form of cryo-injury during cryopreservation is the formation of ice crystals during the procedure which can cause osmotic injury and ultrastructural damage to the cell (Litvan, 1972; Karlsson & Toner, 1996; Taylor & Fletcher, 1999; Gao & Critser, 2000; Day & Brand, 2005). Ice crystals can be formed in either the extracellular or intracellular matrix during the freeze/thaw process (Taylor & Fletcher, 1999; Day & Brand, 2005; Chang & Zhao, 2021), and this is dependent on the rate of cooling (rapid or slow) (Mazur *et al*., 1972). Extracellular ice crystals are formed during slow cooling (-1℃/min), and during thawing, between -15℃ and -160℃ (regarded as the lethal temperature zone), the ice crystals can transform and expand, causing mechanical damage to the cell (Taylor & Fletcher, 1999; Gao & Critser, 2000; Chang & Zhao, 2021). Presence of extracellular ice crystals increases the solute concentration in the cell which causes osmotic stress and eventual cell death (Chang & Zhao, 2021).

Intracellular ice crystals are formed during fast cooling (Day & Brand, 2005). At a fast cooling rate (-200℃/min and greater), intracellular ice crystals are formed due to supercooling which does not allow an osmotic equilibrium to be attained (Karlsson & Toner, 1996; Gao & Crister, 2000). During the thawing process these ice crystals can recrystallize and expand due to ice

transformation during the lethal temperature zone, leading to cryo-injury and eventual cell death (Meryman, 1971; Mazur, 1984; Karlsson & Toner, 1996; Chang & Zhao, 2021).

Cell dehydration

Cooling of cells at a sufficiently slow rate (-1℃/min) allows for water to move out from the intracellular matrix without supercooling (Karlsson & Toner, 1996; Taylor & Fletcher, 1999; Gao & Critser, 2000). This results in the cell undergoing dehydration and thereby preventing ice formation intracellularly (Karlsson & Toner, 1996; Taylor & Fletcher, 1999; Gao & Critser, 2000). However, cell dehydration causes cell shrinkage and an increase in solute concentration that results in cellular damage within the cell (Gao & Critser, 2000). Cell membranes are weakened by such dehydration as it affects the lipid-protein complexes of the phospholipid bilayer (Karlsson & Toner, 1996; Taylor & Fletcher, 1999; Gao & Critser, 2000). A weakened cell membrane loses its selective permeability, resulting in an influx of electrolytes that can cause the cell to swell and burst post-thaw (Karlsson & Toner, 1996; Gao & Critser, 2000). To overcome these effects the protocol for cryopreservation plays a vital role.

1.2.3. Cryopreservation Methods and Protocols

Various cryopreservation methods and protocols, such as vitrification, have been developed to mitigate cryo-injury (Taylor & Fletcher, 1999). Vitrification is a process in which the viscosity of the intracellular matrix is elevated preventing the formation of ice crystals resulting in a glass-like intracellular matrix, which decreases the amount of available water for ice crystal formation (Fahy *et al.*, 1984; Day *et al*., 2000; Chang & Zhao, 2021). To achieve vitrification, the algal cell must be dehydrated before cooling by adding a highly concentrated cryoprotectant which interacts with the intracellular water and reduces the possibility of ice-crystal formation (Fahy *et al.*, 1984; Rall & Fahy, 1985; Karlsson & Toner, 1996; Taylor & Fletcher, 1999; Day *et al.*, 2000; Chang & Zhao, 2021).

For cells that are normally found in an aqueous environment there are three main factors that determine successful cryopreservation: the rate of cooling, cryoprotective additives, and the rate of thawing.

Rate of Cooling

Cooling rates are either rapid or slow but within each category there are several variations (Karlsson & Toner, 1996; Cañavate & Lubian, 1997; Day *et al*., 2000). The rapid cooling method involves the direct immersion of the cell into liquid nitrogen at -196℃, thereby cooling at a rate of -200℃/min (Karlsson & Toner, 1996; Cañavate & Lubian, 1997a; Day *et al.*, 2000). In this method, cooling occurs rapidly preventing an osmotic equilibrium between the intracellular and extracellular matrix, and depending on the sample water content, the cells can freeze without the formation of ice crystals (Fuller *et al*., 2004). This results in a higher solute concentration in the extracellular matrix, and the osmotic flow of water from the intracellular matrix – thereby decreasing the amount of available water to crystalize (Day & Brand, 2005). Whilst this rapid approach may work well for some higher plant species, very few algal species have been reported to survive the stress of such cooling (Taylor & Fletcher, 1999; Abreu *et al*., 2012; de Jager, 2015). Cooling at a slower rate has been identified as ideal for microalgae (Table 1). Slow cooling methods allow the cells to 'adjust' osmotically to the lowering temperatures (Taylor & Fletcher, 1999) (Table 1). In this regard, the two-step cooling method has specifically been used extensively for microalgae. This method gradually cools samples from ambient temperature to -30℃, at a constant rate of -1℃/min, before plunging into liquid nitrogen (-196℃) (Cañavate & Lubian, 1997a; Taylor & Fletcher, 1999, Day *et al*., 2000; Abreu *et al*., 2012). This gradual cooling allows for cell dehydration, preventing ice crystal formation and ultrastructural damage (Gao & Critser, 2000; Day & Brand, 2005).

Cryoprotective additives (CPA)

Cryoprotective agents are substances that protect cells from cryo-injury by reducing/preventing intracellular and extracellular ice crystal formation (Meryman, 1971b; Fahy *et al*., 1990, Fahy, 2010). There are two types of cryoprotectants, those that permeate the cell to equilibrate the extracellular and intracellular matrix (penetrative CPA), and non-permeating ones that act in the extracellular matrix (non-penetrative CPA) by increasing osmolarity, thereby dehydrating the cell prior to freezing (Fuller *et al*., 2004; Day & Brand, 2005). Penetrative cryoprotectants are usually chemicals that are highly soluble in water and can cross cell membranes rapidly equilibrating in the cytoplasm - examples of these are glycerol, dimethyl sulfoxide (DMSO) and methanol (Meryman, 1971b; Hubalek, 2003; Fuller *et al*., 2004). These cryoprotectants are low in cellular toxicity and impart freeze tolerance by lowering the freezing point of the cells (Fuller *et al*., 2004). However, to successfully achieve a vitreous solution for cryopreservation, the cryoprotectant concentration is expected to be high enough to replace intracellular water (Sakai & Engelmann, 2007), and this high concentration could cause cytotoxicity (Sakai & Engelmann, 2007). The use of penetrating CPAs is also important for the maintenance of salt/solute concentrations that can alter when ice is formed as they act as a secondary solvent for salts, thereby decreasing the amount of ice formed at a certain temperature (Fuller *et al*., 2004). Consequently, penetrative CPAs reduce cryo-injury at low temperatures using the slow cooling method (Fuller *et al*., 2004; Fernandes *et al*., 2019; Paredes *et al*., 2020).

Empirical experimentation has shown that several types of cryoprotectants can be applied to microalgae. DMSO is one of the most successful and hence, widely utilised cryoprotectants (Paredes *et al*., 2020). DMSO interacts with intracellular water to depress the intracellular freezing point, and is also an intracellular electrolyte modifier, phospholipid bilayer protector and a free radical scavenger (Rammler & Zaffroni, 1967; Yu & Quinn, 1995; Lee & De Mora, 1999). The protection of the phospholipid bilayer by DMSO is crucial during cryopreservation.

Addition of DMSO causes the cell membranes to become “floppier” and allows for the phospholipid bilayer to be more accommodating to water molecules by inducing water pores, thereby preventing osmotic and mechanical stress during cryopreservation (Notman *et al*., 2006). DMSO also protects the electron transport chain (Lee & De Mora, 1999) and the enzymes responsible for oxidative phosphorylation (Dickinson *et al*., 1967), localised to the inner membrane of the mitochondrion. This form of protection is important during the freezing steps of the cryo-procedure, to prevent oxidative stress. DMSO has also been regarded as the most effective cryoprotectant for the cryopreservation of mitochondria (Greiff & Myers, 1961), with the caution that it should be removed as quickly as possible post-thaw as it can alter the permeability of the inner mitochondrial membrane and interfere with the organelle’s oxygen uptake (Fuller *et al*., 1989). The introduction of DMSO beyond a threshold can cause cytotoxicity, as these compounds are foreign to the cells (Yu & Quinn, 1995). Determining an appropriate concentration and exposure time to the cryoprotectant is therefore vital, as species will respond differently to varying concentrations of cryoprotectants.

Rate of Thawing

Injury during cryopreservation procedures is not exclusive to freezing. Cells are exposed to the lethal intermediate temperature zone again during thawing, which can cause recrystallization (Gao & Critser, 2000). Recrystallization is the change in phase from vitreous that formed during cooling to crystalline ice, causing cryo-injury (Gao & Critser, 2000). To ensure that recrystallisation does not occur during thawing, it is recommended that cells be thawed rapidly at rates between 0.25℃ to 16℃/min to ensure a high post-thaw viability (Cañavate & Lubian, 1995a).

In addition, cryoprotectants can be cytotoxic post-thaw (Fuller *et al*., 2004), and to prevent this they should be diluted immediately after thawing (Fahy, 1986; Fahy *et al*., 1990; Cañavate & Lubian, 1995a). Cell death due to photo-oxidative stress can also occur post-thaw, due to the

production and accumulation of ROS (Tatone *et al*, 2010; Chen *et al*., 2015). To counteract this, cells should be incubated in the dark immediately after DMSO dilution (Day & Brand, 2005).

The stress responses of cells to cryopreservation have been extensively characterised (Fig. 1.6) and provide insight on all factors that need to be taken into consideration when finding a suitable cryopreservation protocol.

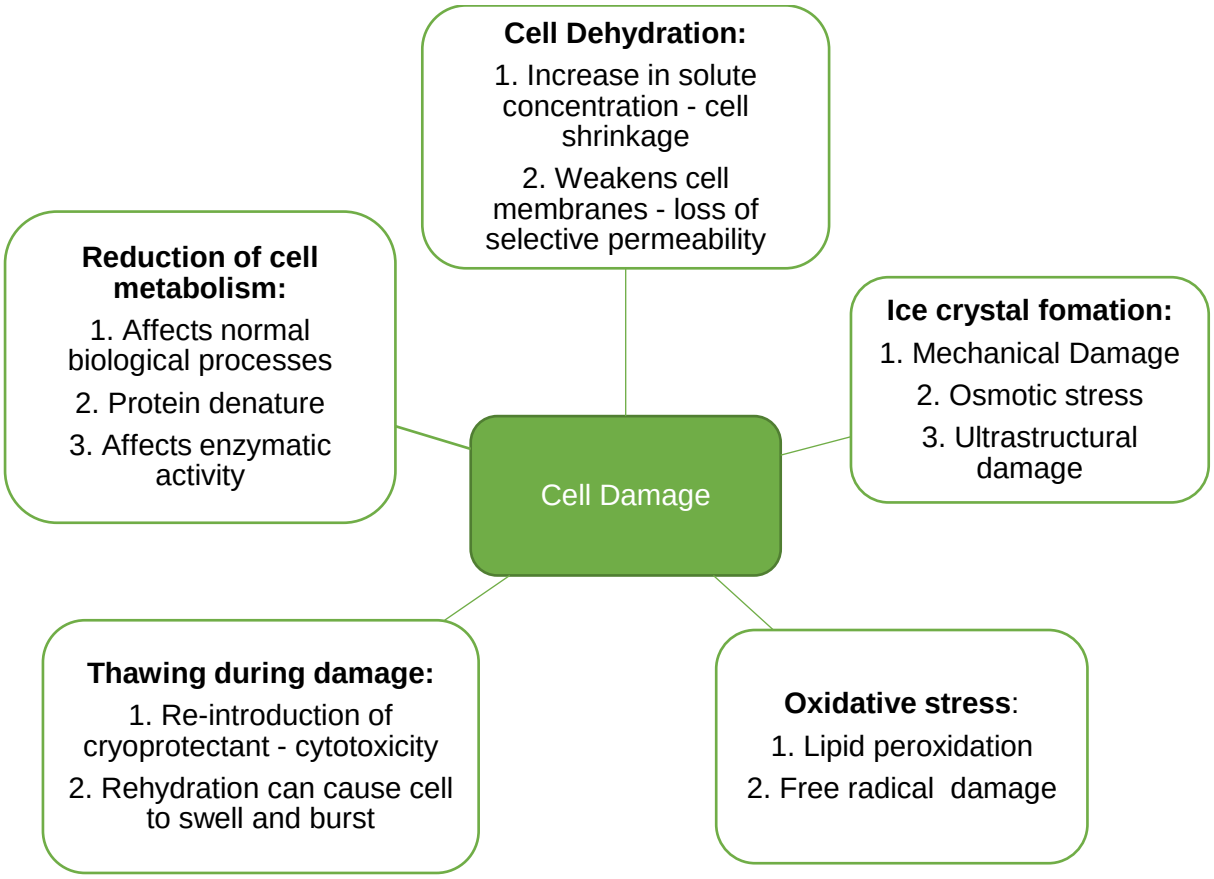


Figure 1.6: Summary of the main causes or types of cell damage during the cryopreservation process.

1.2.4 Effects of Cryopreservation on Mitochondrial function

To have an effective cryopreservation protocol, it is important to know how the procedure effects mitochondrial activity as the organelle plays a vital role in both cell survival (aerobic respiration) and death (e.g., ROS production). Mitochondrial functioning is therefore an indication of the ability of the cells to survive the various cryo-procedures and to re-establish in culture. Rapid cooling (~800 °C/min) without the use of a cryoprotectant was suitable for the freezing of mitochondria, with little to no damage to their membranes (Araki, 1977). Slow cooling during freezing causes more damage to mitochondria (Grieff & Myers, 1961; Lusena, 1965; Sherman, 1971), but this could be overcome by the addition of DMSO (Grieff & Myers, 1961; Fuller *et al.*, 1989).

Inner mitochondrial membrane potential is established by carrier proteins pumping protons from the matrix into the intermembrane space (Becker *et al*., 2009). The potential is reduced by allowing some of the protons back into the mitochondrial matrix, at the same time driving the ATP synthase in the inner membrane to make adenosine triphosphate (ATP) (Becker *et al*., 2009). Thus, mitochondrial membrane potential is indicative of an active and functional mitochondrion.

Various methods have been used to understand the effects of cryopreservation on mitochondrial function. Early research focused on measuring oxidative phosphorylation by measuring oxidative enzymes (Grieff & Myers, 1961; Araki, 1977), or by looking at respiration and ATP production (Fuller *et al*., 1989). Recently, fluorescent staining techniques have been used and are regarded as a reliable measure for viability (Johnson *et al*., 1981; Garner *et al*., 1994; O'Connell *et al*., 2002; He & Wood, 2004) and mitochondrial activity (Cottet-Rousselle *et al.*, 2011). Hence this technique will be used to assess viability and mitochondrial function during the cryopreservation procedure.

1.2.5 Effect of Cryopreservation on Cell Ultrastructure

Cryo-procedures have implications on cell functioning at varying levels. The physical and physiological implications have been studied intensively for microalgae (Day & Fleck, 2015), but research on ultrastructural effects is limited. From the studies conducted to date, freezing stress causes membranes to lose selective permeability, resulting in organelle enlargement (particularly the chloroplast), increased vacuolisation and eventually cell death (Morris, 1978; Nagao *et al*., 2008). However, addition of a cryoprotectant to the cryo-procedure results in the reduction in chloroplast size, the formation of small vacuoles and the modification of the membrane structure from one that is liquid crystalline to one that is gel-like (Plattner *et al.*, 1972; Morris *et al*., 1977). The most crucial alteration post-thaw, is the loss of selective permeability in the plasma membrane, leading to cell death (Steponkus, 1985). Hence, the focus was on membrane integrity, specifically the plasma membrane, chloroplast envelope and thylakoids, and mitochondrial membranes.

1.3. Rationale

Successful cryopreservation protocols (cryoprotocols) have been identified for several microalgal species (Table 1), but the application of such protocols cannot be standardized across the greater microalgal genetic diversity (Taylor & Fletcher, 1999; Day & Brand, 2005).

There are currently four known studies that have been conducted on the cryopreservation of *Pyramimonas*. One was on *Pyramimonas gelidicola*, a psychrophilic (cryophilic) vestigiferan species from Antarctica with anti-freezing properties (Hong *et al.*, 2011), and this species was able to be successfully cryopreserved using the fast cooling method (direct plunging into liquid nitrogen) and could survive DMSO exposure at concentrations up to 30 % for 10 minutes. Two studies (de Jager, 2015; Mokoena, 2017) focused on developing an appropriate protocol for the genus. The first of these worked on *Pyramimonas pseudoparkae* (*Trichocystis*) and

Pyramimonas mucifera (*Punctatae*); and while the latter used four species, each representing one of the four supported subgenera of *Pyramimonas* (Daugbjerg *et al*., 1994; Viprey *et al*., 2008). The vitrification method using 10 % DMSO in combination with the slow cooling method was appropriate for the cryopreservation of some species belonging to the subgenera *Trichocystis*, *Punctatae* and *Vestigifera* (de Jager, 2015; Mokoena, 2017). Lastly a study on three species, from subgenus *Vestigifera* – *Pyramimonas orientalis*, TAK 25 (*P. sp.*) and *Pyramimonas mitra* (Liu, 2018), found that viability of these species decreased with increasing concentration of DMSO (10 – 20 %) and exposure time (8 – 60 min), with low recovery rates (<50%). The varied results obtained for this genus highlight that a standard protocol would not be easy to achieve, hence the importance of optimising cryopreservation protocols.

The cryoprotectant DMSO together with the two-step cooling method have been appropriate for some representatives of the Chlorophyta (Table 1, Appendix). The appropriate technique to successfully cryopreserve these species has been identified (de Jager, 2015; Mokoena, 2017), and that the technique is practicable for the preservation of some of these fragile organisms. However, the biological responses to the stresses of preparation, freezing and recovery are still not understood. Thus far, research focus has been on achieving the actual protocol for cryopreservation rather than considering other aspects such as decreased cell viability at each of the separate preparative procedures. An understanding, at the cell biology level, of the impact of cryopreservation can inform the direction of future development of more efficient protocols. To address this, the impact of the cryoprotectant, and the cumulative impacts of the freezing and thawing processes on the viability of, and ultrastructure in *Pyramimonas mucifera* will be examined.

Pyramimonas mucifera was subject in one of the forementioned studies and has been selected as a model species, due to the varied response to previous attempts at cryopreservation (de Jager, 2015; Mokoena, 2017). In one study, the post-thaw viability for this species was <15 %

(de Jager, 2015), and 100 % recovery in the other (Mokoena, 2017). Hence, the need to understand its biological response via membrane integrity and mitochondrial function. The biological response attained will demonstrate the suitability of cryopreservation as a conservation method for this species.

1.4. Aim and Objectives

The aim of this study was to investigate the effect of cryopreservation, using 10 % DMSO and the two-step cooling method on the viability and ultrastructure of *Pyramimonas mucifera.*

To achieve this aim, the following objectives were followed:

1. To determine cell viability of *Pyramimonas mucifera* using membrane integrity, with the use of fluorescent stain Propidium Iodide (PI), after treatment with 10 % DMSO and the two-step cooling method
2. To determine the mitochondrial function of *Pyramimonas mucifera* after treatment with 10 % DMSO and the two-step cooling method, using fluorescent stain Rhodomine 123 (Rh123).
3. To characterize the ultrastructural response of *Pyramimonas mucifera* to 10 % DMSO and the two-step cooling method, with a focus on the plasma membrane, the chloroplast, and the mitochondrion.

2. Methods and Materials

The experimental design wass divided into different stages, as see in Figure 2.1 below. The cultures were prepared for use before preliminary investigations, treated with DMSO and cryopreserved using the two-step cooling method (Fig. 2.1). Viability was assessed at different stages of the experimental design using either PI or Rh 123. Ultrastructural investigations occurred after DMSO treatment and post-thaw after a 13-day recovery period.

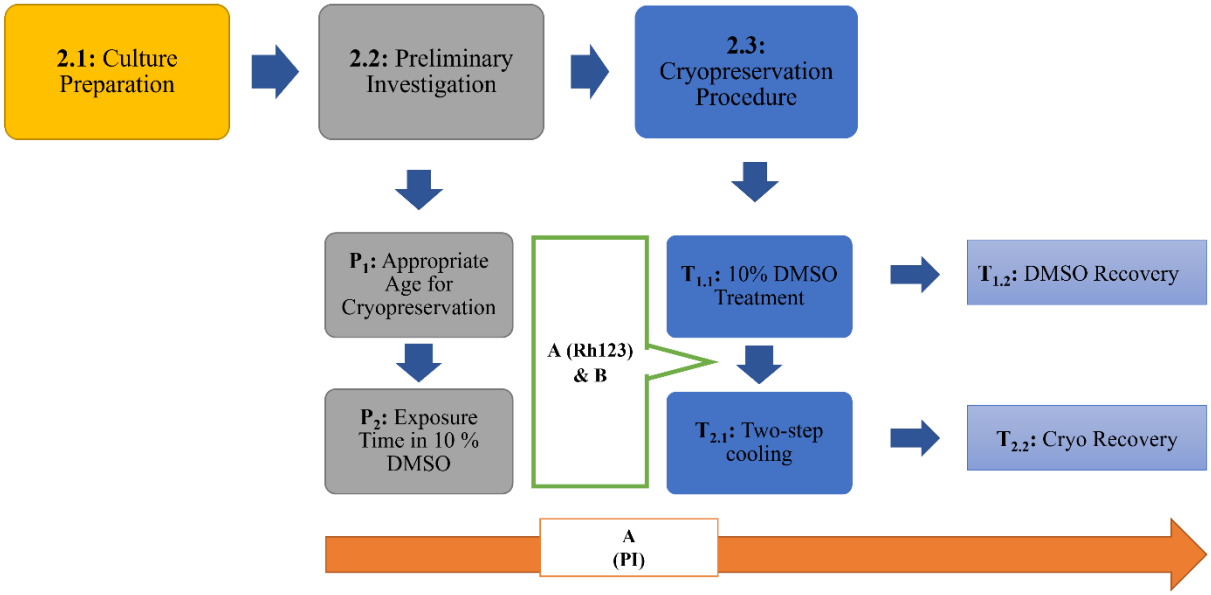


Figure 2.1: Schematic representation of the experimental design. **2.1** Culture preparation for experimental use. **2.2** Preliminary investigations for determining the appropriate culture age (**P_1**) and exposure time (**P_2**) to 10 % DMSO. **2.3** Cryopreservation procedure. **A**. The stages in the process for viability and mitochondrial function (MF) assessments using PI and Rh123 (staining reagents). **B**. Ultrastructural investigations in the experimental process. **T_1**: DMSO treatment and recovery (7 days). **T_2**: Two-step slow cooling method, thawing and recovery (13 days).

2.1 Culture Preparation

A clone of *Pyramimonas mucifera* (UW DE.A4) was obtained from the algal collection housed at the University of the Witwatersrand, Johannesburg, South Africa. Stock and experimental cultures were established by inoculating approximately 2 ml healthy stock culture into 100 ml IMK-enriched (Diago, Niahon Pharmaceutical Company, Japan) autoclaved seawater in 250 ml Erlenmeyer flasks. Stock cultures were sub-cultured every 14 days to ensure nutrients were not depleted. The cultures were grown at 20 ℃ and at 330 µmol. Photons $m^{-2}s^{-2}$, with a (14:10) day/night cycle.

2.2 Preliminary Investigations

P_1: Appropriate Age of Culture for Cryopreservation

Cultures in the exponential to early stationary phase were at the optimal stage for cryopreservation as their cells were not stressed, still rapidly dividing, and were sufficiently concentrated to maximize cell encounter (Day & Brand, 2005). Using a Neubauer haemocytometer (Day & Brand, 2005; Lois & Siegal, 2011) and a Zeiss 46 70 650 – 9905 compound light microscope, a growth curve was constructed by counting the number of cells in an aliquot of 20 µl, in ten replicate cultures, daily over a twenty-day period. These counts were then used to calculate the cell density per sample per day and the growth curve was plotted. Cells were gently swirled to resuspend cells that may have settled to the bottom of the culture vessel. Viable cells were those that had retained their shape, flagella, and integrity. Non-viable cells were those that had lost their shape, flagella or had burst/ lysed. The following equation was used to determine cell density for the growth curve:

$$d = (D.F/n * x) * 10\,000$$

d = cell density; D.F = dilution factor; x = average count per square; n = number of squares counted.

P_2: Exposure Time in 10 % DMSO

DMSO was previously determined as an appropriate cryoprotectant (de Jager, 2015). A concentration of 10 % was found suitable for successfully cryopreserving *P. mucifera* compared to 5 % and 15 % DMSO (Mokoena, 2017), thus this DMSO concentration was used in this study. Exposure time to 10% DMSO was, however, still unknown. To determine the appropriate exposure time of the cells to the cryoprotectant, 10 culture samples were exposed to 10 % DMSO at 1:1 ratio for 30, 45 and 60 minutes in the dark. The untreated controls were also diluted at 1:1 ratio with culture medium for comparison. The behaviour at the various time intervals was observed and cell viability (via plasmalemma integrity) was assessed using PI. The sample was gently swirled before sampling to suspend *P. mucifera* cells which preferred to settle at the bottom of the culture vessel (see section 1.1). This was done throughout experimentation.

2.3 Cryopreservation Procedure

Samples of the algal culture were used for viability and ultrastructural assessments before and after treatment with DMSO, and following each of the DMSO recovery, thawing and cryo-recovery periods. Ten replicates were used for the cryo-procedure.

$T_{1.1}$: Treatment with 10% DMSO

The samples were treated with 10 % DMSO for 30 minutes (section 2.2) in the dark to ensure cellular penetration and to prevent oxidative stress (Steffen & Palta, 1989).

$T_{1.2}$: DMSO Recovery

After the desired exposure period, the DMSO was diluted by adding 9 ml of fresh medium to 1 ml of each sample. These were then allowed to recover under normal growth conditions (section 2.1).

$T_{2.1}$: Two-step Cooling

One millilitre of each of the cell samples was treated with 10 % DMSO (section $T_{1.1}$) and from the resultant culture, a 1 ml aliquot was added to each of ten Nunc vials. To facilitate the slow cooling (~ 1 ℃ per min), the Nunc vials were placed in Nalgene® Mr. Frosty, filled with isopropyl alcohol and stored overnight in a -80 ℃ refrigerator. The isopropyl alcohol allows gradual cooling at -1 ℃/min. The following day the Nunc vials were removed from Mr Frosty and rapidly transferred into liquid nitrogen, at -196 ℃ for a minimum of 45 minutes.

$T_{2.2}$: Thawing and Cryo-recovery

For thawing, the Nunc vials were placed in a 30 ℃ water bath and removed as soon as the sample transitioned into the liquid phase in order to prevent heat-associated cellular damage post-thaw. The DMSO-enriched algal culture was then serially diluted by adding 500 µl IMK-enriched (Diago, Niahon Pharmaceutical Company, Japan) autoclaved seawater to the solution every minute. Gentle swirling took place between each aliquot addition until a 6 ml solution was amassed. The culture was equilibrated in the dark for 30 minutes and then further diluted by the addition of 20 ml of autoclaved seawater medium. The samples were left for 13 days – this was regarded as the cryo-recovery step – after which viability was assessed (see below). This period was required to allow surviving cells to multiply and to maximize the encounter of cells when viewed with the microscope.

A. Viability & Mitochondrial Function Staining Analysis

Poly-l-lysine was used to immobilize cells on Teflon-welled slides for fluorescent microscopy. Poly-l-lysine was smeared on the slides, immediately rinsed with ultra-pure water and left to dry. Once dry, the cell sample was transferred to the slide and left to settle for 10 minutes. Following this, the cell sample was incubated in PI and Rh123 (see viability & mitochondrial function assessment below). Fluorescent staining with PI and Rh123 was used to determine

whether the cells were alive or dead, as well as to determine if there were changes in mitochondrial function between treatments. After incubation, the slides were rinsed in seawater twice to remove any excess stain. The coverslip was sealed with nail varnish to prevent desiccation of the sample. The samples were viewed with an Olympus BX63 OFM microscope and images captured using an Olympus DP 80 camera. The various filters used for fluorescence microscopy are provided below.

Viability Assessment

PI was used to determine cell viability and cell membrane integrity. PI is a fluorescent stain that interacts with nucleic acids, but is impermeant to live cells (Zamai *et al*., 1996; William *et al*., 1998). This allows PI to be used to determine cell viability by distinguishing between intact and damaged plasma membranes, i.e., live/dead cells. A stock solution of PI was made by dissolving 0.2 mg of PI in 1 ml of distilled water, which was then stored at 4 ℃ in the dark until used. To determine cell viability during the cryopreservation procedures, the culture samples were incubated at a 9:1 ratio with PI for 10 minutes at 20 ℃. The maximum excitation wavelength for PI was 493 nm and the maximum fluorescence emission wavelength was 488nm.

The following equation was used to calculate cell density for fluorescence viability using PI:

$$d = (D.F * x) * {}^{1000}/_{a}$$

d = cell density; D.F = dilution factor; x = number of cells counted per well; a = number of cells in 90 µl.

Mitochondrial Function Assessment

The fluorescent stain used as a marker for mitochondrial activity was Rh 123. This stain can permeate the cell membrane of living cells and is used to show changes in potential across mitochondrial membranes (Chen, 1988; Ferlini *et al*., 1995). An accumulation of Rh 123 in the mitochondrion is indicative of mitochondrial activity and results in a green-fluorescent staining of the organelle (Chen, 1988; Cottet-Rousselle *et al.,* 2011). A low fluorescent intensity is indicative of dissipation of membrane potential (Cottet-Rousselle *et al.,* 2011). This can be related to a decrease in mitochondrial activity and possible cell death. A stock solution of Rh 123 was prepared by dissolving it in 99.9 % DMSO at the rate of 1 mg/ml and then stored in the dark at -5 ℃. To determine mitochondrial function during the cryopreservation procedures, the culture samples were incubated in Rh 123 in a 9:1 ratio respectively for 10 minutes at 20 ℃. The maximum excitation wavelength for Rh 123 was 617 nm and the maximum fluorescence emission wavelength was 534nm. The intensity of fluorescence of Rh123 in 100 *P. mucifera* cells per treatment was quantified using Image J (Schindelin *et al*., 2012) as described by Hammond (2014) and portrayed as Corrected Total Cell Fluorescence (CTCF). The relative fluorescent intensity was grouped into the following ranges of relative fluorescent unts (RFU): -100 000 to 0; 1000 to 100 000; 100 000 to 200 000. These ranges were used to determine the trends in fluorescent intensity of the different treatments relative to the untreated control. This indicated whether or not the cells had lost mitochondrial membrane potential.

B. Ultrastructural Investigation – TEM Preparation

Transmission electron microscopy of thin sections of fixed material was used to determine whether there were ultrastructural alterations to the cells following the various stages of processing (Fig. 2.1). The cells were prepared for electron microscopy using the Na-cacodylate fixation method used by Inouye *et al.,* (1990). A culture sample of 9.5 ml was fixed in 0.5 ml of 25 % glutaraldehyde, in 0.1 M Na-cacodylate buffer containing 0.5 M sucrose (hereafter

referred to as “buffer”) for 1 hour at room temperature and centrifuged for 15 minutes. The pelleted cells were then washed four times in 0.1 M Na-cacodylate buffer with decreasing sucrose concentrations (from 0.5 M to 0 M). Thereafter, the pellet was post-fixed for 1 hour in 2 % osmium tetroxide (OsO_4) in buffer, followed by another wash in buffer. Cells were dehydrated with increasing alcohol concentrations (10 %, 20 %, 50 %, 80% and 90 %) for 15 minutes in each solution, followed by two washes in 100 % alcohol for 20 minutes each. The cell samples were then embedded in Spurr’s epoxy resin (Spurr, 1969) and polymerised for 16 hours in a 70 ºC oven. Ultrathin sections (approximately 60 nm) were obtained using an RMC Cryo-Ultramicrotome and placed on copper grids for viewing. These sections were stained with uranyl acetate and lead citrate, viewed using a FEI Tecnai F12 Transmission Electron Microscope and photographed with the TVIPS TemCam-F416 camera.

The electron micrographs were used to determine if there were any obvious ultrastructural alterations between treatments. Attention was specifically paid to the overall cell morphology, integrity of the plasma membrane, various membranes of the chloroplast, and mitochondrion. To prevent bias, the first twenty cells encountered in the linear transect of the grid were used for analysis.

2.4 Data Analysis

Microsoft Excel 2010 and R studio (R Core Team, 2018) were used for statistical analyses for each aspect of the investigation (statistics were not performed on recovery data, as they are binary (living or dead)):

A Shapiro-Wilk test was used throughout to test for normality. A One-way ANOVA was used when the data were normal, and Kruskal-Wallis one-way analysis of variance test was used for non-normal data.

P_2: Exposure Time in 10 % DMSO

A one-way ANOVA was used to determine whether there was an interaction between DMSO exposure time at the different intervals, and a Tukey HSD test was used to determine where these interactions occurred. The Kruskal-Wallis one-way analysis of variance test (Hollander & Wolfe, 1973) was used to determine whether there was a significant difference in the viability levels at the various treatment times. A Kruskal Multiple Comparison test (Siegal, 1957) was then used to determine exactly which treatments were significantly different.

A. Viability Assessment & Cell Density

The Kruskal-Wallis one-way analysis of variance test (Hollander & Wolfe, 1973) was used to compare the cell density and cell viability across all five treatments. A Kruskal Multiple Comparison test (Siegal, 1957) was then used to determine where the differences occurred between the different treatments.

Mitochondrial Function

Image J (Schindelin *et al*., 2012) was used to quantify the intensity of fluorescence across treatments for mitochondrial function analysis (Hammond, 2014). The Kruskal-Wallis one-way analysis of variance test (Hollander & Wolfe, 1973) was then used to determine if there was a significant difference between mitochondrial membrane potential across the treatments. A Kruskal Multiple Comparison test (Siegal, 1957) was used to show were the differences occurred.

B. Ultrastructural investigation

Image J (Schindelin *et al*., 2012) was used to: i) measure cell length & width; ii) analyse membrane integrity (particularly of the plasma membrane, chloroplast, and mitochondrion); iii) and note any vacuolization in the cells. The Kruskal-Wallis one-way analysis of variance

test (Hollander & Wolfe, 1973) was used to compare changes in cell size between treatments. The Kruskal Multiple Comparison test (Siegal, 1957) was then used to demonstrate where these differences occurred.

3. Results and Discussion

3.1. Preliminary Experimentation

P_1: Appropriate Age of Culture for Cryopreservation

The late exponential/early stationary phase of the growth of *Pyramimonas mucifera* cultures was between day 13 and 14 (Fig. 3.1).

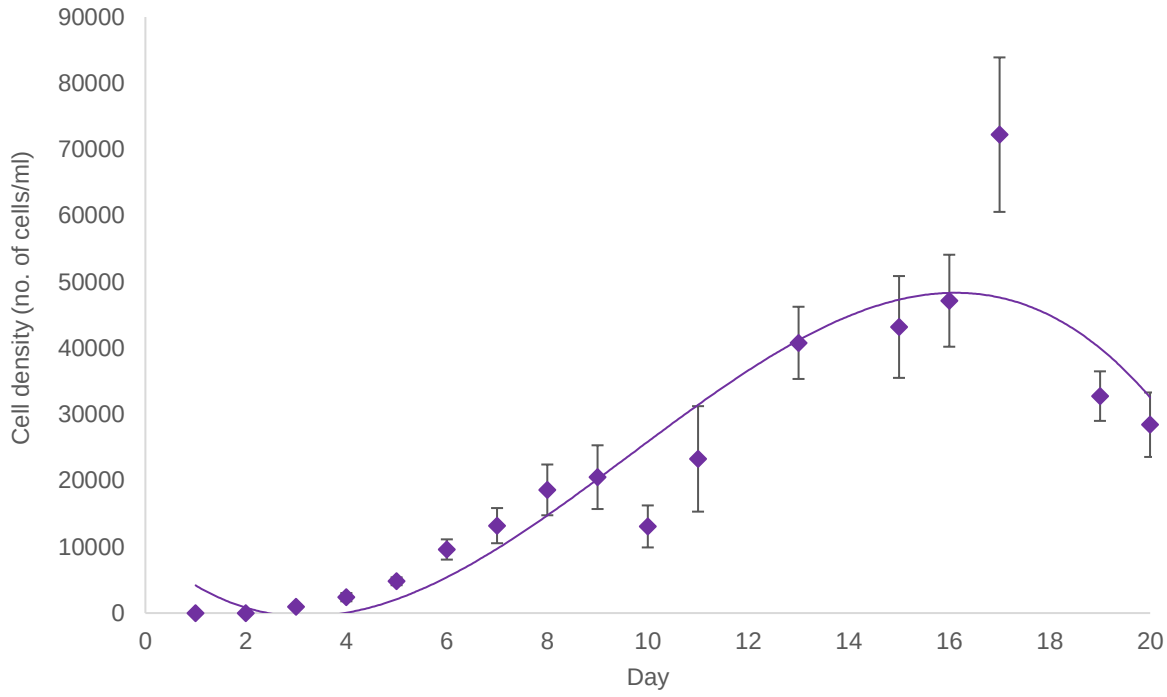


Figure 3.1: Growth Curve for *Pyramimonas mucifera* over a 20-day period

P_2: Exposure Time in 10 % DMSO

To determine whether DMSO was an appropriate cryoprotectant, it was important to consider its concentration and exposure period. This was to ensure cell viability was not compromised before exposure to the cryoprocess. Microalgal species have different tolerance levels to both the cryoprotectant and freezing (Cañavate & Lubian, 1994; Joseph *et al.*, 2000). It was therefore important that the effect of each was determined for any species investigated. In the

case of *P. mucifera*, 10 % DMSO had been previously determined as the appropriate concentration (Mokoena, 2017), however, the optimal exposure time was still unknown. To determine this, *P. mucifera* cells were treated in 10 % DMSO for varying times up to 1 hour.

In the early stages of exposure (< 15 minutes) to DMSO, cells were sluggish and did not swim as freely as those in the untreated culture medium. However, cell shape and integrity were both maintained. Cells survived DMSO exposure up to 1 hour (Fig. 3.2 & 3.3). Cell deformation was observed after only 30 minutes of exposure. Beyond this, movement had stopped but cell shape and integrity were maintained. After 1 hour of exposure, cell membranes remained intact, but cell and chloroplast shape had been compromised. Although exposure of cells to DMSO (regardless of the period) affected percentage viability, the surviving cells re-established their culture population within 7 days of its removal (Fig. 3.3). The type of extracellular coverings affect the exposure period to cryoprotectants (CPAs) in some microalgal species (Boroda *et al.*, 2014). These coverings are diverse across microalgal species and based on their different compositions, can either be rigid or non-rigid (Domozych *et al*., 2012). Rigid cell coverings are impermeable to CPAs (Boroda *et al.*, 2014). In this regard, some microalgae species, such as *Tetraselmis chuii* and *Tetraselmis gracilis*, are able to withstand prolonged exposure (~ 4 hours) to DMSO (Cañavate & Lubian, 1994; Joseph *et al*., 2000) due to the presence of a rigid theca made of fused scales (Domozych, 1984). Cells of *P. mucifera* do not have a theca and are only protected by layers of discrete scales (Sym & Pienaar, 1991). The scale coverings in this species are porous and do not form a rigid boundary like a theca, allowing immediate exposure of the plasmalemma to DMSO. Thus, these cells were expected to be more sensitive to prolonged periods (> 30 minutes) of DMSO exposure than walled forms. The observed loss in cell shape and integrity is evidence of this sensitivity, as is the decline in percentage viability (Fig. 3.3). To ensure that *P. mucifera* cells are not compromised by DMSO cytotoxicity, a period of 30 minutes exposure was deemed appropriate for successful cryopreservation.

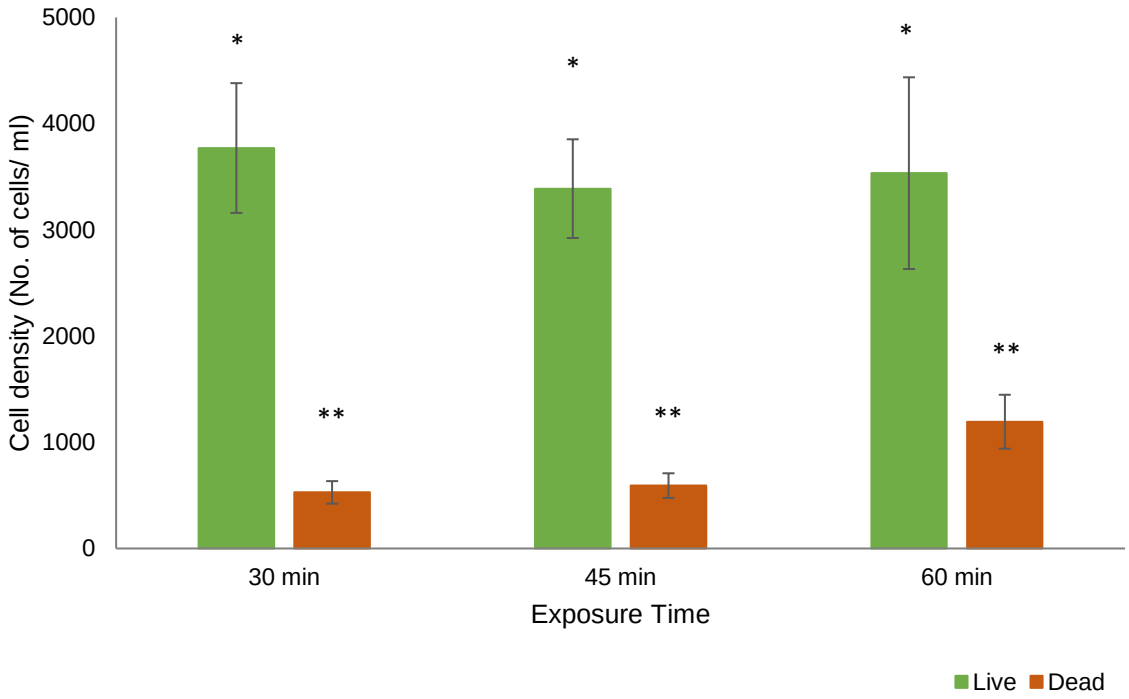


Figure 3.2: Cell density of *P. mucifera* cells (live and dead) exposed to 10 % DMSO for 30, 45 and 60 minutes. Live: One-Way ANOVA F= 0.08, *df* = 2, $p > 0.05$; Dead: Kruskal-Wallis chi-squared = 5.0584, *df* = 2, $p > 0.05$. * Shows significance between live cells and ** between dead cells.

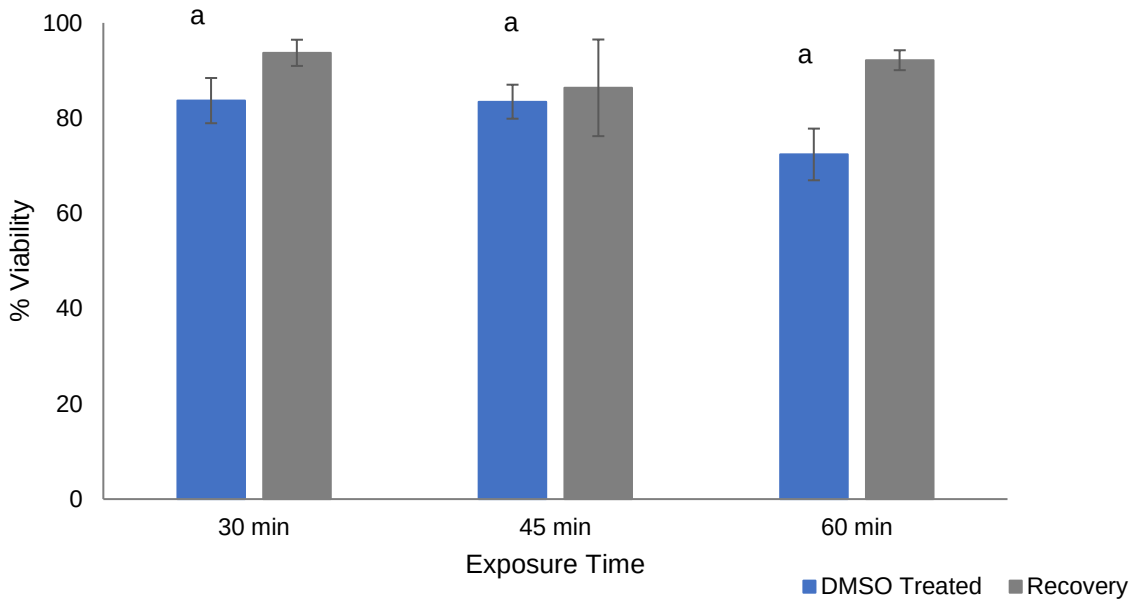


Figure 3.3: Percentage viability of cells of *P. mucifera* cells exposed to 10 % DMSO for 30, 45 and 60 minutes. The DMSO was then diluted out of the cells and the cells were allowed to recover over a 7-day period. Percentage viability was recorded following staining with PI. Kruskal-Wallis chi-squared = 5.5877, $df = 2$, $p > 0.05$. Treatments with different letters are significantly different.

3.2. Cryopreservation Procedure

All cultures of *Pyramimonas mucifera* subjected to 30 min of 10 % DMSO were successfully cryopreserved (Table 2).

Table 2: Sample recovery after treatment in 10% DMSO and the two-step cooling method for cryopreservation.

	NO. OF SAMPLES	***NO. OF SAMPLES RECOVERED***	***% RECOVERY***
DMSO RECOVERY	10	10	100
CRYO RECOVERY	10	10	100

A. Viability Assessment – Cell Membrane Integrity

Previous studies on the cryopreservation of microalgae predominantly determined cell viability post-cryopreservation through culture reestablishment, colony formation (cell culturing on a substrate such as agar) and cell counting using a haemocytometer (Cañavate & Lubian, 1994, 1995a; Fenwick & Day, 1992; Joseph *et al*., 2000; Rhodes *et al*., 2006; Guermazi *et al*., 2010). These techniques were not always reliable measures when cells were exposed CPA's (Pozzobon *et al.,* 2020). The use of fluorescent PI staining as a viability assay is a better method than those previously mentioned (Pozzobon *et al.,* 2020), and was used as a more definitive measure of viability.

T_1: Treatment with 10% DMSO & Recovery

After 30 minutes exposure to 10 % DMSO, 88 % of the cells were viable when stained with PI (Fig. 3. 4). After a 13-day recovery period, the cell density and viability increased (Fig. 3.4 & Fig. 3.5) showing that after DMSO dilution, the cells can recover and re-establish their

populations in culture. This means that DMSO is a suitable CPA for *P. mucifera* cells and had minimal effect on cell membrane integrity (Fig. 3.5).

T_2: Two-step Cooling and Cryo Recovery

Successful cryopreservation of various microalgae to date includes the use of the two-step cooling method (Ben-Amotz & Gilboa, 1980; Gwo *et al.*, 2005; Tanniou *et al*., 2012; Paredes *et al*., 2020). Cryopreservation on the viability of cells already reduced by exposure to DMSO was monitored. The cell density (94 % drop) and viability (61 % drop) significantly decreased immediately post-thaw compared to their values after DMSO exposure (Figs 3.4 & 3.5).

In addition, the exposure to cryoprotectants and to the freezing and thawing processes induces stress and has been related to cryo-recalcitrance (Day *et al*., 2000) and the triggering of apoptosis (Jiang *et al*., 2019). The cryopreservation of *P. mucifera* did not trigger apoptosis in the surviving cells.

Whilst it is informative to have some knowledge of the minimum cell density to recover viable cultures post-cryopreservation, the current protocol used cultures of optimal age where cell density exceeded this threshold. This was demonstrated by all cryopreserved material recovering to dense cultures after 13 days (Fig. 3.5). This negated the need to determine the threshold itself, but for our understanding, its determination would be interesting, and it is recommended that it be investigated in future.

Pyramimonas gelidicola (KOPRI AnM0046), has been successfully cryopreserved at a higher DMSO concentration (30 %) for up to 10 minutes, using the rapid cooling method (Hong *et al*., 2011). The success of this method in *P. gelidicola* could be due to the presence of ice binding proteins (IBPs) that have allowed the species to survive such ultra-low temperatures (-195.8 °C) (McFadden *et al*., 1982b; Hong *et al*., 2011). These IBPs are presumed to have been acquired by horizontal gene transfer from cryophilic bacteria to enable their survival in this

habitat (Raymond & Kim, 2012). On the other hand, *P. mucifera* is found in tidal pools in the Western Cape, South Africa (13 to 24 °C) (Sym & Pienaar, 1991) and have no chance of withstanding such extremes. The direct plunging / fast cooling method was unsuccessful in this species (de Jager, 2015).

A distinguishing characteristic of this species is the presence of mucilage (extracellularly) and muciferous vesicles (intracellularly) (Sym & Pienaar, 1991). In normal conditions, to allow attachment to the substrate, mucilage is secreted and is composed of a mixture of proteins and polysaccharides (Sym & Pienaar, 1991). The presence of these vesicles decreases the amount of free and available intracellular water in the cells, thus lessening the chances of ice crystal formation. Successful cryopreservation with higher plant cells (e.g., Cactus species: *Opuntia ficus- indica (L.), Opuntia humifusa,* and *Opuntia streptacantha*) with intracellular (Tao & Li, 1986; Goldstein & Nobel, 1994) and extracellular mucilage located between the plasmalemma and the cell wall in those plant cells have been regarded as an effective ice-crystal-free buffer zone (Olien, 1981). *P. mucifera* cells in benthic populations, could also be producing the same mucilaginous buffer zone as in higher plant cells. This buffer zone could be acting as a natural cryoprotectant, thereby assisting DMSO in preventing cryo-injury and increasing the possibility of cryosuccess.

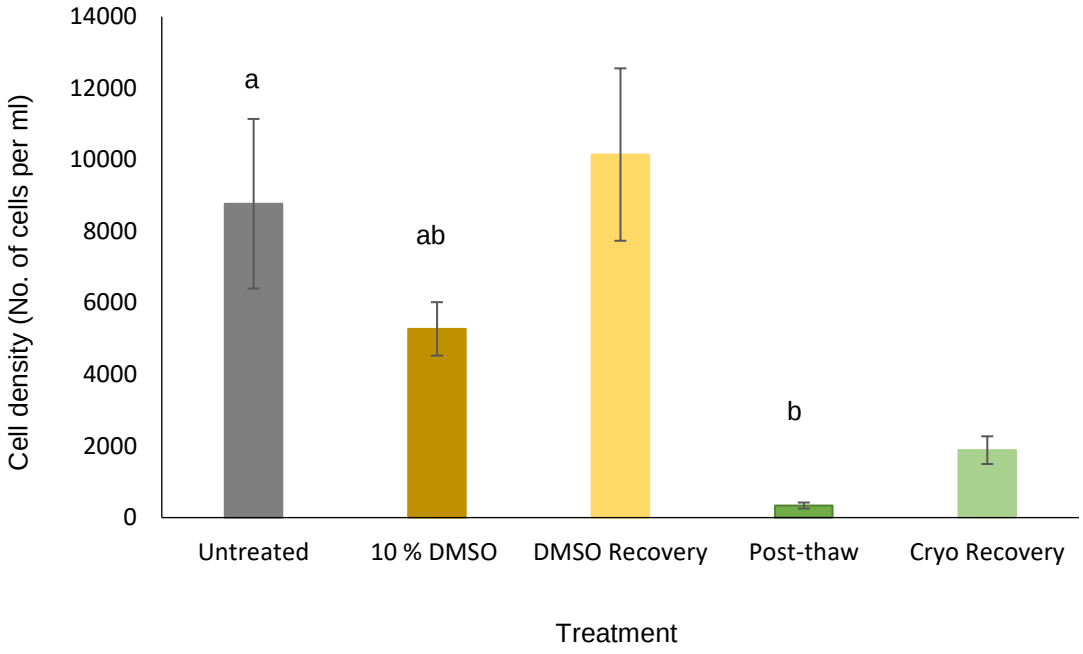


Figure 3.4: The fate of density of *P. mucifera* cells in samples before treatment, following 30 min exposure to 10 % DMSO, following thaw after cryopreservation and finally after a 13-day recovery period. Kruskal-Wallis chi-squared = 19.577, $df = 2$, $p < 0.05$. Treatments with different letters are significantly different.

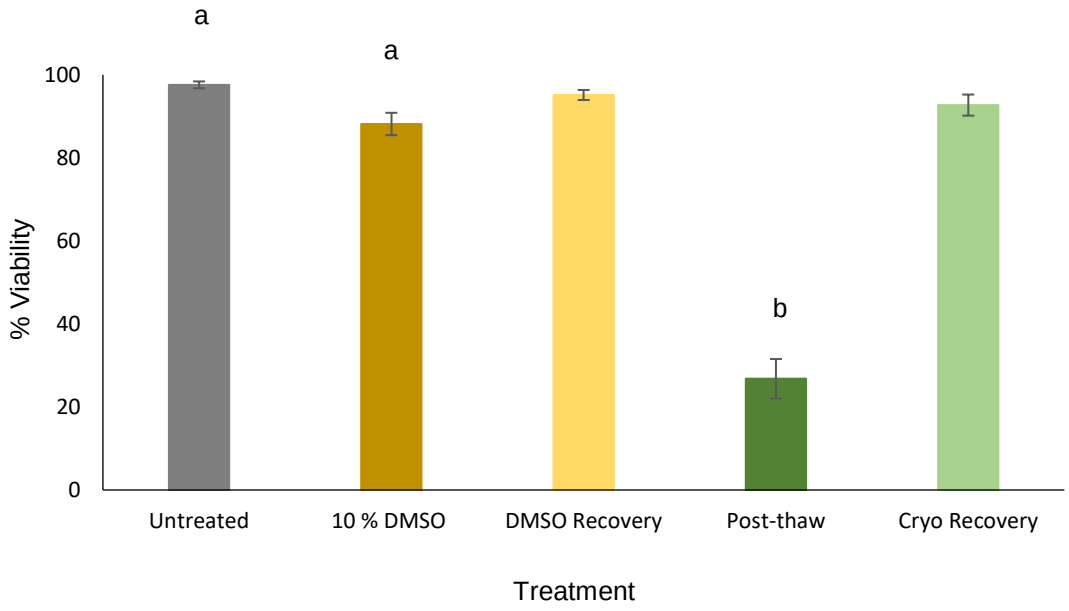


Figure 3.5: Percentage viability of *P. mucifera* cells before treatment, following 30 min exposure to 10 % DMSO, following thaw after cryopreservation and finally after a 13-day recovery period. Cell viability was calculated as the percentage of live cells using PI as the viability stain. Kruskal-Wallis chi-squared = 24.603, $df = 2$, $p < 0.05$. Treatments with different letters are significantly different.

A. Mitochondrial Function

The mitochondrial membrane potential of untreated cells had a fluorescence range from 1 000 to 200 000 RFU.

T_1: Effect of 10% DMSO

During the 30-min exposure to 10% DMSO, the fluorescent intensity of *P. mucifera* cells significantly decreased to half that of the control (Fig. 3.6). This was not surprising as DMSO is an unfamiliar solute to the cells (Fuller *et al.*, 1989; Elliott *et al.*, 2017), and causes toxicity via destabilisation of proteins and enzymes (Fahy *et al.*, 1984) as well as disruption of protein interactions (Arakawa *et al.*, 1990). However, 93 % of the DMSO treated cells fluoresced in

the 1 000 to 200 000 RFU range (Fig. 3.7). Thus, although average mitochondrial activity / membrane potential was lower than that of the untreated *P. mucifera* cells, it remained within the control range indicative of normal mitochondrial activity (Fig. 3.7). This correlated with 88 % of *P. mucifera* cells were viable (using PI; Fig. 3.5) when treated with 10% DMSO, and together these results highlight the ability of DMSO to protect cell membranes during cryopreparation. It can be deduced that 10% DMSO, at 30 minutes exposure, was able to permeate the mitochondrial membranes with little to no effect on normal mitochondrial function, and efficiently protected the mitochondrion from cryo-injury.

T_2: Effect of the Two-step Cooling Method

Immediately post-thaw, mitochondrial membrane potential significantly decreased by 74 % compared with the untreated *P. mucifera* cells and by 47% relative to those treated with DMSO alone (Fig. 3.6). This decline indicated mitochondrial membrane damage may have occurred during the freezing or the thawing process. Slow cooling causes more damage to mitochondrial membranes than rapid cooling (Akari, 1977). However, the addition of DMSO reduces this damage and protects the organelle (Grieff & Myers, 1961). There are two ways that DMSO can damage membranes post-thaw: 1. cytotoxicity due to the DMSO present in the cell once metabolism is restored (Fuller *et al*., 1989), and 2. osmotic injury during thawing, caused by the intracellular accumulation of DMSO, resulting in an influx of water into the cell due to a higher solute concentration within than outside (Elliot *et al*., 2017). This influx of water into the cell interrupts the cell's homeostatic mechanisms and facilitates membrane damage through loss of selective permeability (Day & Brand, 2005; Elliot *et al*., 2017). Although DMSO has many properties that aid successful cryopreservation at temperatures below -22℃, it is important that it is slowly diluted out of the cell to prevent damage to membranes. Cells immediately post-thaw were immobile, and motility was only observed after a 13 day recovery

period. An extended lag phase should be expected before exponential growth for cryopreserved cultures (Paredes et al., 2020). Motility is a direct indication of an active mitochondrion, as it is dependant on ATP production (Becker *et al*., 2009), and lack thereof indicated an impairment in mitochondrial membrane potential (O'Connell *et al*., 2002). Maintenance of membrane integrity during the freeze/thaw process is directly correlated with mitochondrial membrane potential, as mitochondrial membranes need to be fully functional to retain the membrane potential (Becker *et al*., 2009). In the viability results, membrane integrity of *P. mucifera* was compromised post-thaw (Fig. 3.5), which further explained the decline in mitochondrial membrane potential.

Nevertheless, the distribution of fluorescence of *P. mucifera* cells across the fluorescent ranges (1 000 to 200 000 RFU range) post-thaw was similar to that of untreated cells (Fig. 3.7), suggesting that there was mitochondrial activity in 99 % of the cells. However, this distribution was not spread across the fluorescent ranges as with the untreated and DMSO treated cells, where 98 % of cells were found in the 1000 to 100 000 RFU range (Fig. 3.7). This indicates a decrease in fluorescence of the Rh123 stain and the onset of apoptosis in the cells (Cottet-Rousselle *et al.*, 2011). However, the re-establishment of *P. mucifera* cultures (Table 3) showed that cells consistently survived the cryo-procedure.

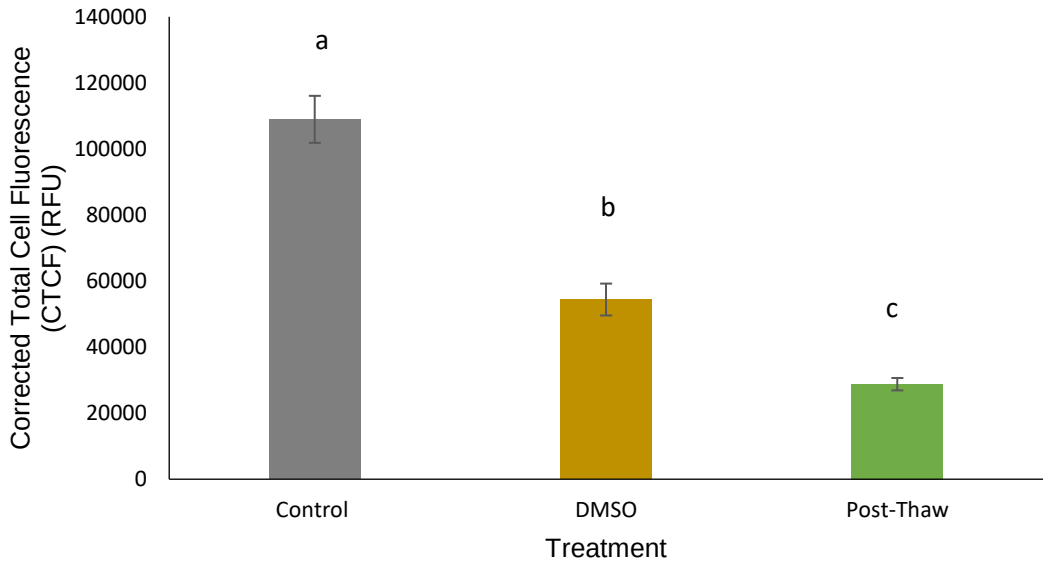


Figure 3.6: Mitochondrial function (determined by Rh123 fluorescent staining) of *P. mucifera* before and subsequent to being subjected to the various steps of cryopreservation. Note the significant decline at each progressive stage monitored. Kruskal-Wallis Non-parametric multiple comparison: Kruskal-Wallis chi-squared = 108.46, df = 2, $p < 0.05$. Treatments with different letters are significantly different.

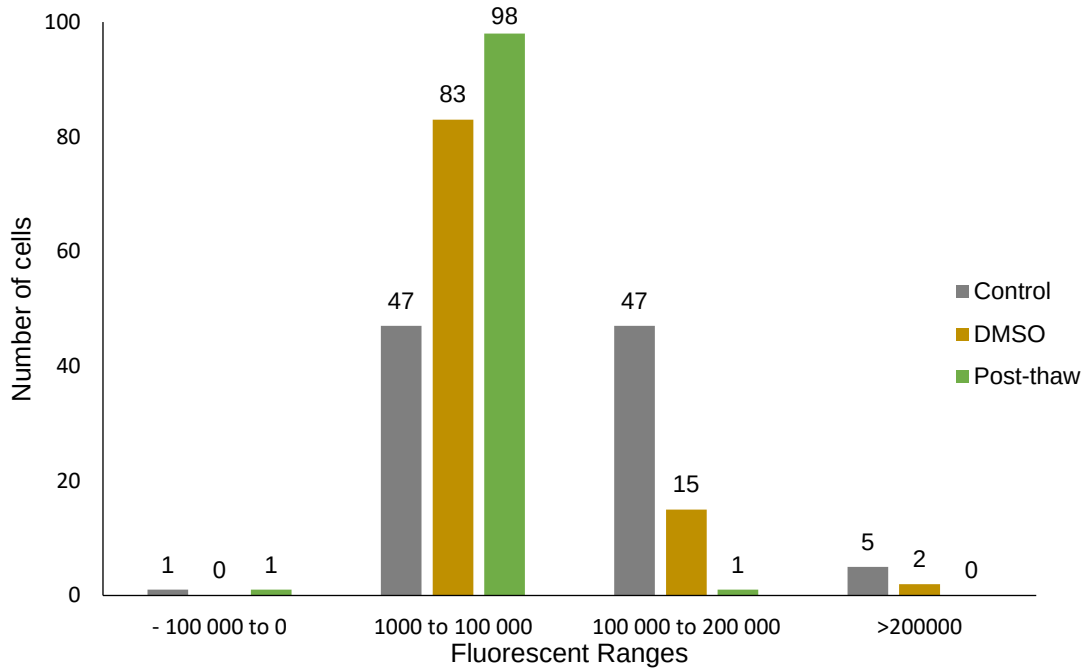


Figure 3.7: Fluorescent ranges of 100 *P. mucifera* cells stained with Rh123 under various treatments (untreated, DMSO, post-thaw). The control cells spread evenly across the 1000 to 200 000 RFU ranges. The 1000 to 100 000 RFU has the highest number of DMSO treated and post-thaw cells, with 17 DMSO treated cells fluorescing in the >100 000 RFU range.

B. Ultrastructural Investigation

T_1: Effect of 10% DMSO

The membrane integrity of *P. mucifera* cells was not affected after 30 minutes exposure to 10 % DMSO. Cell integrity was maintained in all cells, but cell shrinkage was experienced (Table 3). This significant decline in cell dimensions was expected as the addition of DMSO increased the solute concentration in the intracellular matrix and caused the cell to dehydrate (section 1.2.3). Membranes of the cell, chloroplast and mitochondrion did not show any obvious signs of damage (Fig. 3.8). Addition of a cryoprotectant was expected to cause vacuolisation in the cell (Plattner *et al.*, 1972; Morris *et al.*, 1977), however, vacuoles were not seen in the *P. mucifera* cells after DMSO exposure. This suggests that the concentration of DMSO (10 %) did not cause a stress response that would have resulted in its possible

sequestration into vacuoles and emphasised its suitability as a cryoprotectant for this species. This finding correlated with the viability (Fig. 3.5) and mitochondrial function data (Fig. 3.6), and further demonstrated that DMSO was not cytotoxic to *P. mucifera* cells, at least over this period of exposure.

Table 3: Cell measurements (length and width) at different points in the cryo-treatment. Different superscripts represent significant differences between the different treatments.

Measurement / Treatment	***Untreated***	***DMSO***	***Post-thaw***	***Statistics***
Cell Width (µm)	5.74 ± 0.91^{a}	2.82 ± 0.43^{b}	2.51 ± 0.43^{b}	Kruskal-Wallis Nonparametric Multiple Comparison chi-squared = 52.574, df = 2, p-value < 0.05
Cell Length (µm)	6.46 ± 1.05^{c}	2.95 ± 0.32^{d}	2.72 ± 0.36^{d}	Kruskal-Wallis Nonparametric Multiple Comparison chi-squared = 51.474, df = 2, p-value < 0.05.

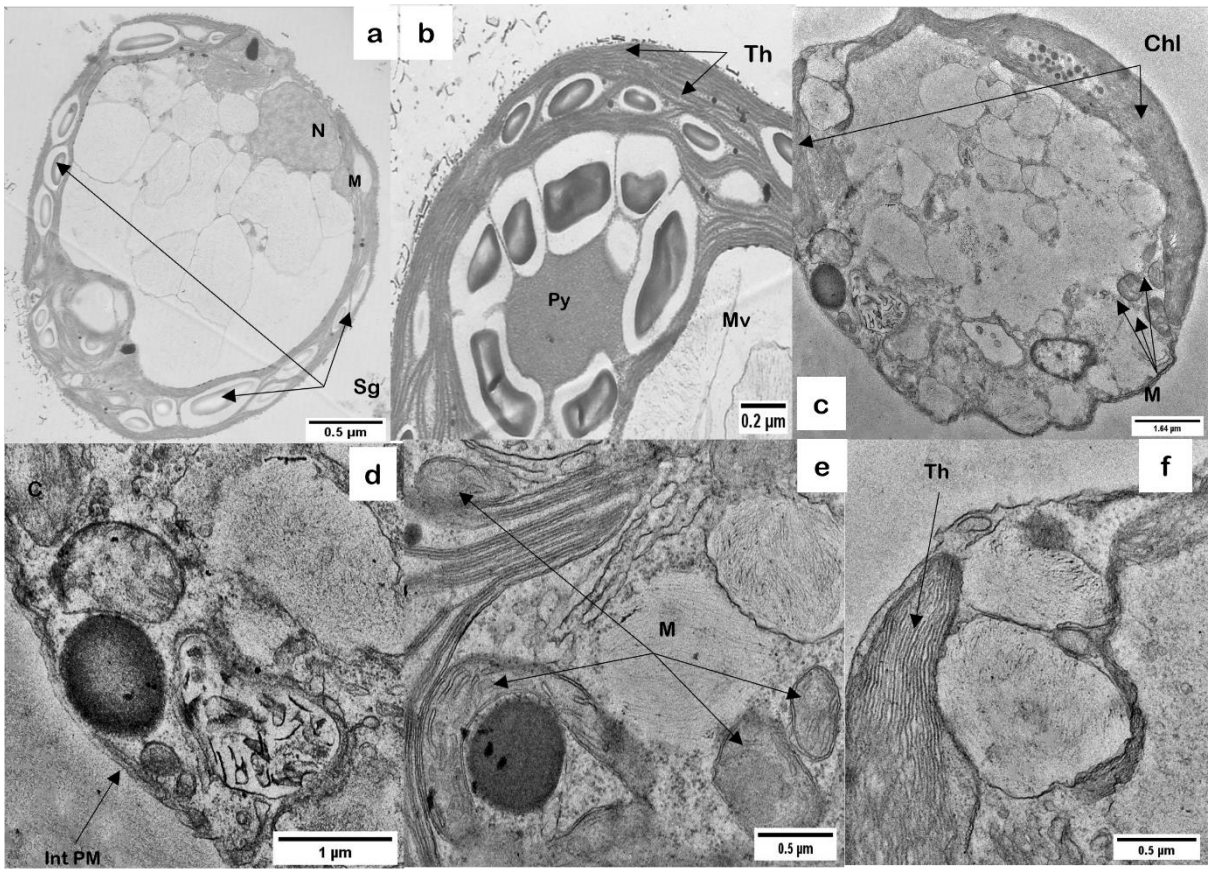


Figure 3.8: *Pyramimonas mucifera* cells after a 30-minute treatment with 10 % DMSO. (a & b): Untreated *P. mucifera* cells. (c – f): Cells treated with 10 % DMSO. No obvious changes in the ultrastructure between the untreated and DMSO treated cells were observed. (c, d & f) Membranes of the chloroplast envelope and thylakoids were intact. (c & e) Mitochondrial membranes remained intact. **Key: C**: chloroplast; **Int Pm**: intact plasma membrane; **M**: mitochondrion; **Mv**: muciferous vesicles; **N**: nucleus; **Py**: pyrenoid; **Th**: thylakoids.

T_2: Effect of the Two-step Cooling Method

Immediately post-thaw, the cell dimensions of *P. mucifera* (Table 3) did not significantly swell or increase in size and were the same as those after DMSO treatment. Thus, selective permeability was maintained, presumably as a consequence of addition of DMSO (Morris & Canning, 1978; Nagao *et al*., 2008). DMSO prevents the weakening of cell membranes and

maintains membrane fluidity, thereby preventing membranes from losing selective permeability (Yu & Quinn, 1995).

The response of *P. mucifera* cells at thawing fell into four categories:

1. Cells that showed evidence of plasmalemma rupture (about one third of the cells encountered) (Fig. 3.9).
2. Cells that maintained plasma membrane integrity, but intracellular structure was compromised (Fig. 3.9). There are two possible explanations for this: 1. a physical distortion due to extracellular ice crystal formation and short-term osmotic stress (Morris & Canning, 1978) or 2. The initiation of apoptosis as described by Darzynkiewics *et al*., 1997 (see Fig. 1.5). Short-term osmotic stress during thawing can cause distortion of the cytoskeleton, resulting in a distortion in the cell's ultrastructure (Morris & Canning, 1978). However, once rehydrated the cell shape would be retained.
3. Cells which demonstrated a clear accumulation of vacuoles relative to the control cells (Fig. 3.9). The presence of vacuoles post-thaw has been reported to be indicative of apoptosis (Affenzeller *et al*., 2009).
4. Cells where membrane rupture was severe, and organelles were structurally compromised (Fig. 3.10). The chloroplast in these cells was fragmented, had lost its shape, and displayed distended thylakoids (Fig. 3.10) as described by Gorelova (2019) for a variety of Chlorophyta species. The mitochondrion did not demonstrate any obvious damage, even in cells that had lost cell integrity and appeared to have undergone cell death (Fig. 3.10). This was not surprising as the mitochondrion plays a vital role in triggering apoptotic and nonapoptotic cell death and is often the last organelle to fragment (Green & Reed, 1998; Green & Kroemer, 2004; Tait & Green, 2013).

From the above observations, it can be concluded that the cryopreservation protocol caused both necrotic and apoptotic death in some *P. mucifera* cells. However, two-thirds of the cells maintained plasmalemma integrity and cell ultrastructure after the freeze/thaw process, highlighting the ability of DMSO to vitrify the cytosol at low temperature and protect cellular membranes from cryo-injury. The suitability of the two-step cooling method (following the addition of DMSO) in cryopreserving *P. mucifera* was also accentuated.

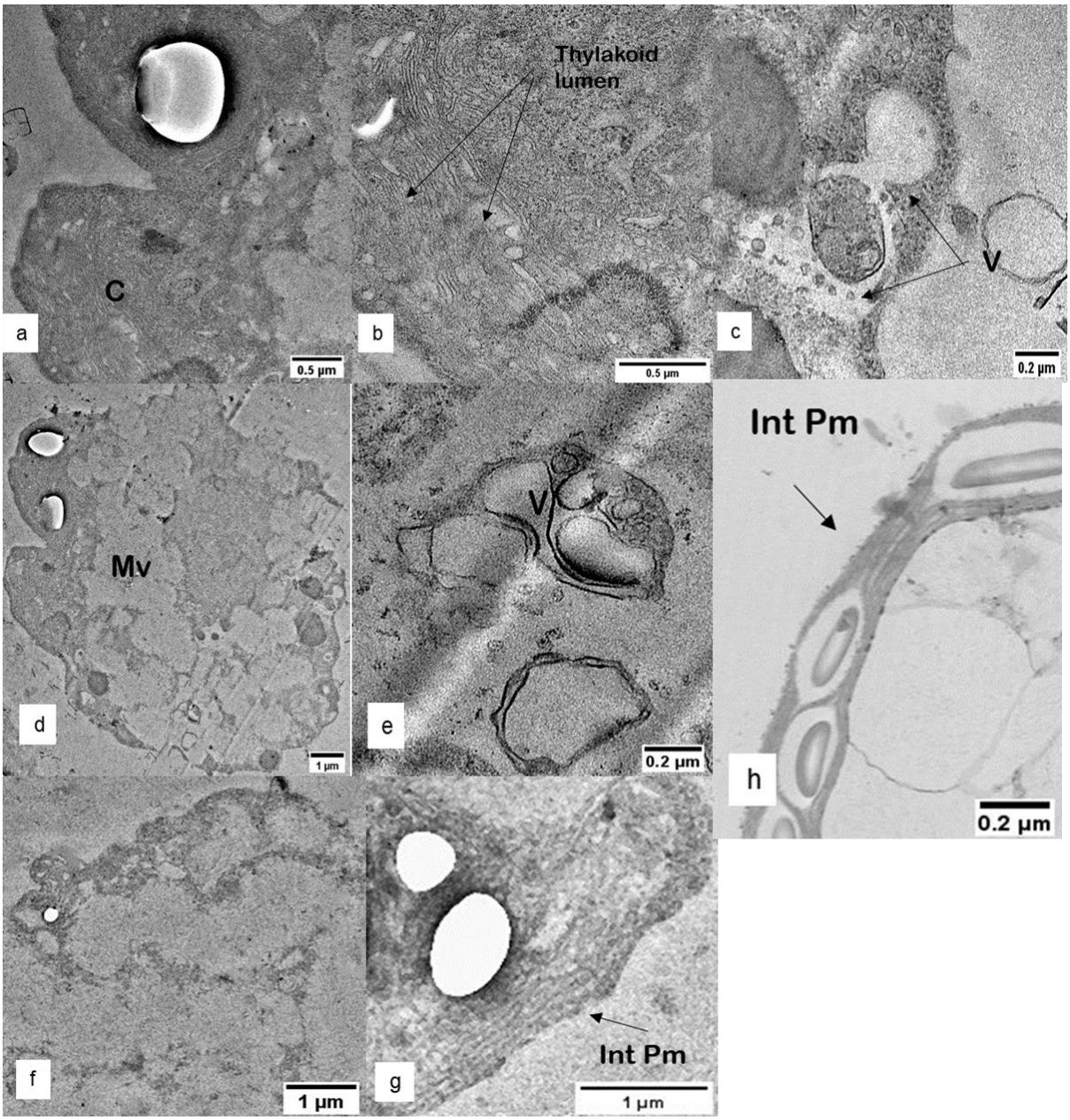


Figure 3.9: *Pyramimonas mucifera* cell after cryopreservation. Cell shape was lost but plasma membrane integrity was maintained. (a & b) Deformed chloroplast in a *P. mucifera* cell fixed immediately post-thaw, with distended thylakoid lumina. (c & e) Vacuolization near the plasma membrane. (d) Entire profile of a cell post-thaw demonstrating loss of cell integrity. (f) Compromised intracellular integrity. (g) Intact plasma membrane. (h) Untreated micrograph of *P. mucifera* cell with intact plasma membrane and chloroplast. **Key: C**: chloroplast; **Int Pm**: intact plasma membrane**; Mv**: muciferous vesicles; **N**: nucleus; **V**: vacuole.

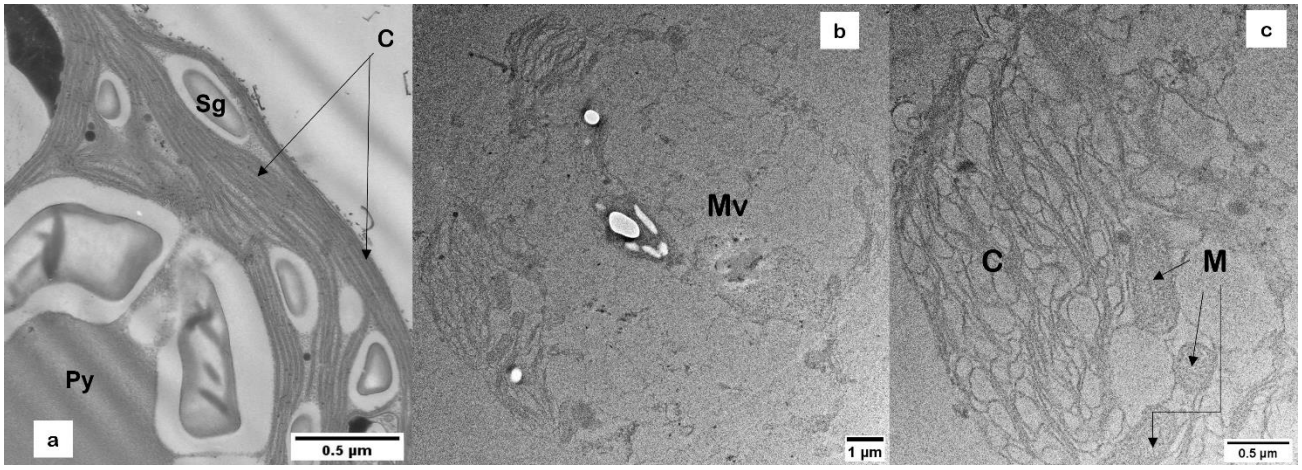


Figure 3.10: *P. mucifera* cells showing mechanical damage, with loss in cell shape and chloroplast integrity. (a) Chloroplast of an untreated *P. mucifera* cell showing the pyrenoid and starch granules. (b) *P. mucifera* cell post-thaw, which has lost cell shape and integrity. (c) Intact mitochondrion, loss of chloroplast shape and expansion of thylakoid lumen in the *P. mucifera* cell post-thaw. **Key: C**: chloroplast; **M:** mitochondrion; **Mv**: muciferous vesicles; **Py** = pyrenoid; **Sg** = starch granules.

4. General Discussion and Conclusion

The individual effects of each step of the cryopreservation protocol for *P. mucifera* was investigated and discussed. To better understand the overall effects the combined achieved results need to be understood.

Effect of DMSO

DMSO was proven appropriate for *P. mucifera* cryopreservation based on viability, mitochondrial function, and ultrastructural response. *Pyramimonas mucifera* cells were viable after a 30 minutes exposure to 10 % DMSO and did not lethally compromise the membrane integrity or ultrastructure. Mitochondrial function was affected by the DMSO; however, it was within the range of normal functioning of untreated cells and did not cause cytotoxicity or cell death. Importantly, after a serial dilution of DMSO, in all the replicates sufficient cells survived to re-establish cultures.

The combined effect of DMSO and the two-step cooling method

Pyramimonas mucifera cells that exhibited signs of mechanical damage, had experienced cryo-injury in the form of ice crystals formation (Day & Brand, 2005) and DMSO toxicity post-thaw (Fuller *et al*., 1989). Ice crystal formation in combination to the DMSO presence in the cell, causes damage during thawing by disrupting the osmotic gradient which causes the cell to swell and will eventually lead to rupture of membranes (Day & Brand, 2005). To prevent this, it is imperative to rapidly thaw the cells and serially dilute the DMSO immediately post-thaw. Evidence of apoptosis was also seen in the mitochondrial function and ultrastructure for *P. mucifera* cells. This demonstrated that these cells exhibited different stress responses depending on the mechanism of cryo-injury as previously described (section 1.2.3). For more quantitative results when determining mitochondrial function and viability, flow cytometry should be considered in future studies.

Regardless, viable cells were consistently recovered and, more importantly, were able to re-establish into vigorously-growing cultures. This means that the cryo-procedure was effective in preserving this species. The response to both DMSO and the freezing/thawing process varied across individual *P. mucifera* cells in the population. The variation was probably due to the underlying biological status of the cell at the time of exposure to each stress, in particular the cell cycle stage at the point of treatment. Culture age plays a crucial role in successful cryopreservation with cultures that are active and vigorously growing having a higher viability post-thaw (Day & Brand, 2005). Hence the importance of ensuring that the cultures are in optimal condition for cryopreservation (Day & Brand, 2005).

The presence of intracellular muciferous vesicles and extracellular mucilage in *P. mucifera* could well contribute to the tolerance characteristic of this species to cryopreservation. This species exhibits both planktonic and benthic populations (Sym & Pienaar, 1991). To optimise this protocol, future studies should determine if cell viability differs between the two populations during the cryo-procedure. This would affirm the notion that mucilage acts as a 'natural cryoprotectant' in this species, as benthic cells are surrounded by mucilage and planktonic cells swim freely in the water column (Sym & Pienaar, 1991).

Conclusion

Cryopreservation is a method that aims to conserve the cell without any alterations after cryostorage. Both DMSO and the freeze/thaw cycle described here had an effect on the vitality of the *P. mucifera* cells as measured by cell membrane integrity, mitochondrial function, and ultrastructural response. Cell viability and mitochondrial function positively demonstrated the post-thaw survival of cryopreserved cells of *P. mucifera* via the maintenance of membrane integrity. Thus, this cryo-procedure was suitable for the long-term preservation of this species. The 100 % recovery of strongly growing cultures from all replicates of the cryo-procedure tested demonstrated its robustness for this species.

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APPENDIX

Table 4: Successfully cryopreserved microalgae and their cryopreservation methods

Reference	Alga	Rate of Cooling	Cryoprotectant
Leibo & Jones, 1963	*Porphyridium cruentum*	Rapid & Slow cooling	none
Hwang & Horneland,1965	*Anistrodesmus sp.,* *Chlamydomonas reinhardtii* *Chlorella sp.,* *Coccomyxa sp.,* *Euglena sp.,* *Scenedesmus sp*	Slow Cooling	10 % glycerol
Hwang & Hudock, 1971	*Chlamydomonas reinhardtii*	Slow Cooling	5 – 10 % DMSO
Tsuru, 1973	*Chlorella sp.,* *Scenedesmus sp.,* *Nitzshia sp.,* *Phaeodactylum tricornutum*	Rapid Cooling	10 % glycerol & DMSO
Morris, 1976	*Chlorella sp.*	Slow Cooling	2.5 - 5 % glycerol & DMSO

Reference	Alga	Rate of Cooling	Cryoprotectant
Morris, 1978	Selection of Chlorococcales	Slow Cooling	5 % DMSO
Morris & Canning, 1978	*Euglena gracilis*	Slow Cooling	10 % DMSO, ethanol, methanol & PVP 5 % glycerol, glycose
Saks, 1978	*Nitzshia sp.,* *Cylindrotheca sp.,* *Phaeodactylum tricornutum,* *Nannochloris sp.,* *Dunaliella salina*	Slow Cooling	5 % DMSO
Ben-Amotz & Gilboa, 1980	*Phaeodactylum tricornutum*	Rapid & Slow cooling	5 - 20 % DMSO
McLellan, 1989	Diatom selection	Slow Cooling	5 - 15 % DMSO, glycerol & methanol
Benhra *et al*., 1994	*Scenedesmus subspicatus*	Slow Cooling	10 % sucrose, 5 % PVP 3 % methanol
Cañavate & Lubian, 1994	*Chaetoceros gracilis,* *Nannochloris gaditana,* *Nannochloropsis atomus,* *Rhodomonas baltica,* *Tetraselmis chuii*	Slow Cooling	10 % DMSO 5 % methanol

Reference	Alga	Rate of Cooling	Cryoprotectant
Cañavate & Lubian, 1995b	*Chaetoceros gracilis, Nannochloris gaditana, Nannochloropsis atomus, Rhodomonas baltica, Tetraselmis chuii, Isochrysis galbana*	Slow Cooling	5 - 15 % DMSO, 1 - 5% methanol
Hirata *et al.*, 1996	*Chlamydomonas reinhardtii*	Encapsulation-Dehydration	0.5 M sucrose
Mortain-Bertrand *et al.*, 1996	*Dunaliella salina*	Slow Cooling	3.5 M glycerol
Cañavate & Lubian, 1997a	*Chaetoceros gracilis, Nannochloris gaditana, Nannochloropsis atomus, Rhodomonas baltica, Tetraselmis chuii*	Slow Cooling & Encapsulation-Dehydration	5 – 15 % DMSO
Cordero & Voltolina, 1997	*Chaetoceros sp., Phaeodactylum tricornutum*	Slow Cooling	1 – 10 % DMSO & glycerol, 10 % methanol
Day, 1998	Variety of microalgae	Slow Cooling	5% DMSO 10% glycerol & methanol

Reference	Alga	Rate of Cooling	Cryoprotectant
Day et al., 2000	*Euglena gracilis*	Encapsulation-Dehydration	0.75 M sucrose
Poncet & Véron, 2003	*Nannochloropsis oculata*	Slow Cooling	2.2M glycerol
Tzovenis *et al*., 2004	*Chlorella minutissima,* *Chlorella stigmatophora,* *Isochrysis galbana,* *Dunaliella tertiolecta*	Slow cooling	5, 10% DMSO 10% methanol 10% propylene glycol
Rhodes *et al*., 2006	*Amphidinium carterae,* *Amphidinium trulla,* *Gymnodinium simplex,* *Chrysochromulina simplex,* *Prymnesium parvum,* *Prymnesium parvum f. patelliferum,* *Isochrysis galbana,* *Pavlova lutheri,* *Chaetoceros calcitrans,* *Chaetoceros muelleri,* *Chaetoceros sp.,* *Nitzschia ovalis,* *Chlamydomonas coccoides,* *Porphyridium purpureum,* *Tetraselmis chuii,*	Slow Cooling	15% DMSO

Reference	Alga	Rate of Cooling	Cryoprotectant
	Tetraselmis suecica, *Raphidiopsis sp.,* *Aphanizomenon flos-aquae*		
Day *et al*., 2007	*Chlorella vulgaris*	Slow cooling, encapsulation-dehydration	10% DMSO
Guermazi *et al*., 2010	*Chlorella vulgaris,* *Isochrysis galbana,* *Dunaliella salina*	Rapid & Slow Cooling	10, 15% DMSO
Tanniou *et al*., 2012	*Haslea ostrearia*	Slow Cooling	5, 10, 15% DMSO
Nakanishi *et al*., 2012	*Chlorella vulgaris,* *Nannochloropsis oculata,* *Tetraselmis tetrathele*	Slow Cooling	5% DMSO, 5% EG, and 5% proline mixture