UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

CONSERVAÇÃO DE BIO-RECURSOS ALGAIS NO BRASIL: ESTABELECIMENTO DE UM BANCO CRIOPRESERVADO

Letícia Piton Tessarolli

Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de DOUTOR EM CIÊNCIAS, área de concentração: ECOLOGIA E RECURSOS NATURAIS

Orientador: Prof. Dr. Armando Augusto Henriques Vieira
Co-orientador: Prof. Dr. John Godfrey Day (SAMS – UK)

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“I almost wish I hadn't gone down that rabbit-hole

— and yet — and yet — it's rather curious,

you know, this sort of life!”

Lewis Carrol - Alice in Wonderland

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Resumo

O estabelecimento de uma coleção de culturas é um passo crucial na conservação de microalgas de água-doce, que é um grupo bastante diverso de organismos que desempenha importantes funções e com vasto potencial para a exploração biotecnológica, particularmente na utilização de metabólitos secundários e produção de biodiesel. O método mais tradicional para manutenção de microalgas é a utilização de culturas metabolicamente ativas. Embora coleções venham sendo assim mantidas há décadas, existem relatos de perdas de características dos organismos. Além disso, essa técnica de manutenção limita o crescimento das coleções, devido aos elevados custos com material e funcionalismo, assim como limitações espaciais. A criopreservação, que é a manutenção de organismos em temperaturas ultra-baixas a ponto de que todas as funções metabólicas celulares sejam pausadas, surge, então, como uma alternativa recomendada para a manutenção de coleções de culturas em longo termo. O uso dessa técnica permite que amostras sejam mantidas por longos períodos de tempo com mínimo manuseio, consequentemente reduzindo os custos de manutenção, enquanto mantém a estabilidade de culturas, já que as temperaturas são baixas o suficiente para eliminar a ocorrência de processos químicos intracelulares.

A Coleção de Culturas de Microalgas de Água-doce (CCMA-UFSCar) mantém atualmente cerca de 700 linhagens de microalgas em culturas metabolicamente ativas, número que está alcançando a capacidade suporte para as práticas rotineiras de manutenção, consequentemente limitando também a provisão de culturas, que beneficiam um grande número de laboratórios pelo Brasil. Assim, esse projeto teve como principal objetivo, estudar a viabilidade da utilização da criopreservação como técnica para manutenção de culturas algais nessa coleção. Inicialmente foram obtidos resultados positivos para a aplicação de um protocolo usual de congelamento, particularmente para algas verdes cocóides. Entretanto, organismos maiores e mais complexos ainda apresentam certa resistência ao congelamento, com baixas taxas de recuperação dos organismos, o que pode exigir testes mais aprofundados e adaptação nos protocolos para atender as particularidades de cada cepa. Em paralelo ao estabelecimento do banco criopreservado de microalgas da CCMA-UFSCar, foram desenvolvidos projetos para estudar alguns detalhes do processo de congelamento e armazenamento de organismos em baixas temperaturas, buscando aprimorar o sucesso da implementação dessa técnica.

Os efeitos da presença de organismos contaminantes nas culturas de microalgas na resposta das culturas ao congelamento, e consequentemente na obtenção de culturas viáveis para futuras aplicações, foram verificados para selecionadas culturas unialgais. Foi possível observar que, para a manutenção de culturas não-axênicas, a escolha da solução crioprotetora (CPA), utilizada para proteção das células durante o congelamento, pode ser crucial na recuperação das culturas, já que é preciso encontrar um balanço entre a melhor proteção para células algais enquanto deve ser evitada a proliferação extrema dos

organismos contaminantes, o que pode inibir o crescimento das cepas desejadas após o processo.

Além da sobrevivência direta ao processo de congelamento, já que esse é um processo potencialmente danoso para as células, também foi testada a longevidade de amostras mantidas em diferentes temperaturas, buscando estabelecer o procedimento mais simples e com melhor custo-benefício para a coleção, enquanto mantém a estabilidade das culturas. As temperaturas de manutenção das amostras, assim como o CPA utilizado durante o congelamento, foram significativas para a continuidade da viabilidade das culturas congeladas, e somente a manutenção das amostras imersas em nitrogênio líquido foi suficiente para garantir a sobrevivência de todas as amostras por um período de um ano. Além disso, a continuidade da viabilidade das amostras mantidas imersas em nitrogênio líquido foi observada também para amostras mantidas por até 40 anos, na *Culture Collection of Algae and Protozoa (CCAP)*, localizada no Reino Unido.

O processo de congelamento *per se* é potencialmente danoso para as culturas a ele submetidas, o que pode vir a causar diferenças em funcionalidade e genéticas nas células que foram submetidas a esse processo. Assim, análises bioquímicas e genéticas foram utilizadas para verificar a estabilidade das culturas submetidas a esse processo.

Para garantir o sucesso da implementação de um banco criopreservado de microalgas para a CCMA-UFSCar, além das medidas de viabilidade e estabilidade, é necessário estabelecer protocolos de gerenciamento das amostras e manutenção de rotina. Assim, foi realizado um estudo de caso contando com as experiências adquiridas na CCAP durante 40 anos de história de um banco criopreservado, e como a aplicação dessas técnicas podem beneficiar o controle de qualidade e gerenciamento da CCMA-UFSCar, facilitando a implementação do biobanco criopreservado para essa coleção.

Abstract

The establishment of culture collections is a vital aspect for the conservation freshwater microalgae, which are a very diverse group of organisms with important ecological functions on the ecosystems maintenance and an immense although still sparsely explored biotechnological potential, particularly for the use of metabolites and biodiesel production. The traditional protocol for culture maintenance is the serial subculturing of metabollicaly active cultures. Although strains have been maintained in metabolically active cultures for decades, there are several reports of loss of characteristics for the organisms thus maintained, and an increase in the number of strains could be the cause for logistical challenges on the maintenance regimes and elevated costs with material and functionalism. Cryopreservation, which is the maintenance of organisms at ultra-low temperatures to the point that all cellular metabolic functions are paused, appears as a recommended alternative for the maintenance of long-term culture collections. The use of this technique allows the maintenance of samples for long periods of time with minimal handling, severely reducing the maintenance costs, while maintaining the stability of cultures, which should be stored at ultra-low temperatures to eliminate the occurrence of intracellular chemical processes.

The Freshwater Microalgae Culture Collection (CCMA-UFSCar) currently maintains approximately 700 microalgae strains in metabolically active cultures, which is reaching maximum support capacity for routine maintenance practices. The requirements with cultures maintenance will also, consequently, affect the provision services of the collection, which are the basis of research of a large number of projects throughout Brazil. Thus, the main objective of this project was to test the feasibility of the use of cryopreservation as a technique to maintain algal cultures in this culture collection. For this, initially positive results were obtained for the application of a standard freezing protocol, particularly for green coccoid algae. However, larger and more complex organisms are still recalcitrant to freezing, with low rates of recovery, which will require further research and adaptation of the protocols to meet the particularities of each strain. In parallel to the establishment of the cryopreserved biobank for CCMA-UFSCar, projects were developed in order to further clarify details and consequences of the process of freezing and storing organisms at low temperatures, seeking to improve the success rates for the implementation of this technique.

The effects of the presence of contaminating organisms on the microalgae cultures on the response of strains to freezing, and consequently on the formation of viable and robust post-thaw cultures, were verified a few selected non-axenic strains. It was noticed that the choice of cryoprotectant solution (CPA), used to protect cells during freezing, may be crucial for post-thaw recovery, and it is necessary to search for balance between obtaining the highest possible viability levels and avoiding the extreme proliferation of contaminating organisms, which could lead to inhibition in the recovery and quality of the desired algal cultures after the process.

In addition to direct survival to the freezing process, which is a potentially damaging for cells, the longevity of samples maintained at different temperatures was also tested, aiming to establish the simplest and most cost-effective procedure to ensure the long term survival of frozen samples. It was observed that the storage temperatures, as well as the CPA used during freezing, were significant aspects to guarantee the continued viability of the frozen cultures, and only samples maintained immersed in liquid nitrogen were sufficiently protected against temperature fluctuations, ensuring the survival of all the samples for longer periods. Furthermore, the continuity of the viability for samples maintained immersed in liquid nitrogen was also observed in a study with samples maintained on these conditions for up to 40 years at Culture Collection of Algae and Protozoa (CCAP, UK).

The freezing process *per se* is potentially damaging to the cultures during the process, which might be the cause for functional and genetic differences in cells that have undergone this process. Thus, biochemical and genetic analysis were used to verify the stability of the cultures submitted to the freezing protocol.

To ensure the successful implementation of a cryopreserved microalgae bank for CCMA-UFSCar, in addition to viability and stability measures, it is necessary to establish protocols for sample management and routine maintenance. Thus, a case study was carried out with the experiences acquired in CCAP during the 40 years of the history of a cryopreserved bank, and how the application of these techniques can benefit the quality control and management of CCMA-UFSCar, facilitating the implementation of the cryopreserved biobank for this culture collection.

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AFLP	Amplified Fragment Length Polymorphism
CCAP	Culture Collection of Algae and Protozoa
BRC	Biological Resource Center
CBD	Convention of Biological Diversity
CCMA	Coleção de Culturas de Microalgas de Água-doce
Chl-a	Chlorophyll-a
CPA	Cryoprotective agent
CRF	Controlled rate freezer
DMSO	*Dimethyl sulfoxide*
HCCA	*α-Cyano-4-hydroxycinnamic acid*
IBGE	Instituto Brasileiro de Geografia e Estatística
IDA	International Depository Authority
IUCN	International Union for Conservation of Nature
LN_2	Liquid Nitrogen
MALDI	Matrix Assisted Laser Desorption Ionization
MeOH	*Methanol*
OECD	Organization for Economic Co-operation and Development
Q/A	Quality Assessment
SAMS	Scottish Association for Marine Science
SoP	Standard operation Procedure
TOF	Time of Flight
UFSCar	Universidade Federal de São Carlos
WC medium	Wright's Cryptophyte medium
WDCM	World Data Centre for Microorganisms
WRI	World Resources Institute

CONTENTS

1 General Introduction

1.1 Biodiversity and conservation

The importance of biodiversity and its conservation are constantly the focus of international political discussion and media coverage, particularly in the last few years. The loss of species due to the environment degradation, climate change and other consequences of human interference in nature, might have disastrous consequences, many still to be uncovered (Pimm and Raven, 2000). This is equally relevant in the microbial field, whose organisms are responsible for much of our atmospheric oxygen, underpin the food web and drive global biogeochemical cycling (Vitousek *et al.*, 1997; Falkowski *et al.*, 1998), aspects crucial to the maintenance of life on Earth.

Therefore, attitudes in the conservation of the global diversity of organisms are now a priority, in order to maintain the ecological "order", as well as the economic and commercial potential of ecosystem services (Tilman, 1999; Hooper *et al.*, 2005; Balvanera *et al.*, 2006). For this, the establishment of culture collections of organisms, which can work as Biological Resource Centers (BRCs), can provide important steps for the conservational efforts, securing the *ex situ* survival of organisms and expanding their availability for further research. The availability of live cultures in culture collections can also provide materials for elaborate screenings of species for potential producers of secondary metabolites and other economically important products (Stacey and Day, 2014).

The commercial and scientific values of living organisms are increasingly being recognized and this recognition is endorsed internationally via the Convention of Biological Diversity (CBD) (Janssens *et al.*, 2010). The CBD was a result of the United Nations Conference on Environment and Development, held in Rio de Janeiro in 1992. The objectives of this Convention were to ensure "*the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources, including by appropriate access to genetic resources and by appropriate transfer of relevant technologies, taking into account all rights over those resources and to technologies, and by appropriate funding"* (IUCN, 1992).

For the *ex-situ* conservation of species, the convention states the need to *"establish and maintain facilities for conservation of and research on organisms, preferably in the country of origin of genetic resources"* (IUCN, 1992). This led to the establishment of new BRCs and also reinforces the need for accurate and complete records of depositories, providing information needed by users to allow them to comply with CBD (Janssens *et al.*, 2010).

1.2 Microorganisms

Microorganisms are by definition microscopic living forms of life, which are often taken as living organisms that are <1mm in size. They can be comprised of single cells, filaments, or complex multicellular colonies. This definition includes: viruses, bacteria, archaebacteria, protozoa, algae and fungi. As a group of organisms, they are the "engines" that drive and maintain global ecosystems fulfilling key components on the cycling of nutrients, particularly in aquatic environments, both as primary producers of biomass, responsible for around 46% of total biomass carbon produced (Field *et al.*, 1998) and also in the consumption/decomposition, playing a cardinal role in nutrient cycling in freshwater and marine environments (Arndt *et al.*, 2000; Weisse, 2002). The phytoplankton community is an important fraction of the microbial diversity, and within this group are many ecologically and economically valuable species, which are the basis of extensive aquatic food chains and also play an important role in the water and climate quality regulation and health aspects (Cairns Jr *et al.*, 1993).

The extent of the total microbial diversity on our planet is still unknown and difficult to estimate (Stoeck *et al.*, 2006), and a large portion of these organisms are still waiting for discovery, description and/or further analysis (Šlapeta *et al.*, 2005), and some fundamental roles of organisms are still at risk, owing to the huge gaps that still exist in currently available information. However, modern molecular methods, such as the use of phylogenetic bioinformatics, based on barcoding genes, have allowed huge progress in our understanding of the relationships between the major taxonomic groups (Fig. 1.1).

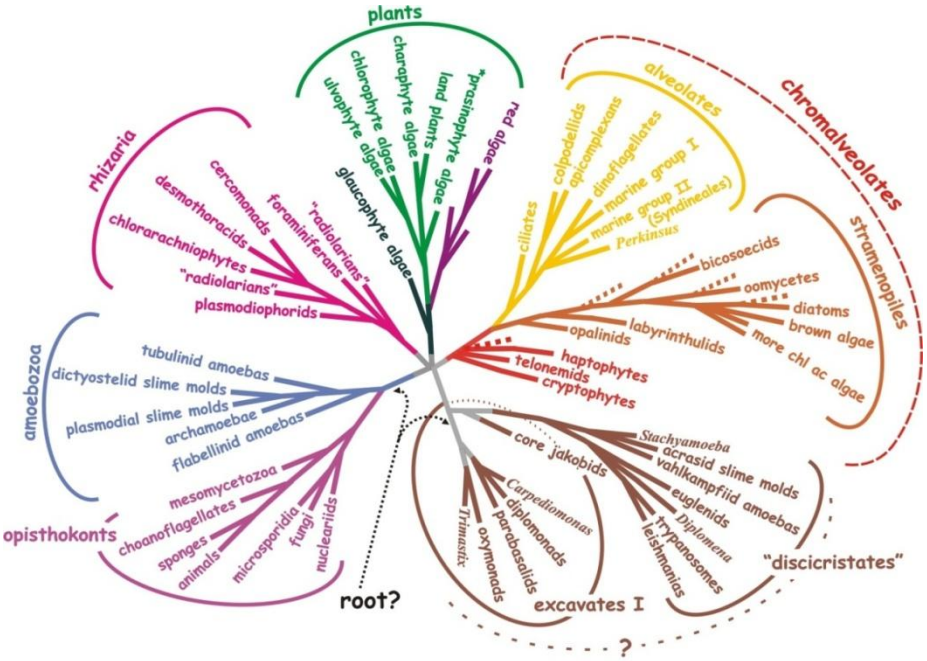

Figure 1.1 - The tree of cellular life
(Baldauf, 2003)

1.3 Microalgae

1.3.1 Microalgae: biodiversity and distribution

The term microalgae may refer to a wide diversity of organisms, ranging from unicellular free-living cells to complex colonial and filamentous organisms (Pulz and Gross, 2004). This group includes both eukaryotic and prokaryotic (mostly) photosynthetic organisms and, due to their polyphyletic origin, they are studied as heterogenic assemblies of organisms without physiological and taxonomic coherence (Andersen, 1992).

These microorganisms are present in all existing ecosystems (Mata *et al.*, 2010) and represent a large component of total biodiversity of inland aquatic ecosystems. However, studies on algal communities' biodiversity, structure and spatial and temporal variation are still limited for some large hydrographic basins.

This group has a large biodiversity, with more than 50.000 described species and genera, widely distributed around the world (Pulz and Gross, 2004). However, their distribution is not uniform, with different richness and diversity in different environments. The

"AlgaeBase" website (http://www.algaebase.org/), is the biggest database dedicated exclusively to the taxonomy and distribution of algae, has now documented in their archives over 40.000 species (Guiry *et al.*, 2014), although some groups are still under represented (Guiry, 2012). The uncertainty on the determination of number of algal species in many groups has important effects on the conservation strategies necessary for this group. Also, the erroneous interpretation of the cosmopolitan distribution within the group might have restricting consequences on the necessary local conservation efforts (Cotterill *et al.*, 2008) as many species of microalgae, particularly the freshwater phytoplankton, are endemic and restricted to a small area (Šlapeta *et al.*, 2006; Taton *et al.*, 2006; Casteleyn *et al.*, 2008), and even small alterations in their habitat could easily lead to the loss of species, including some critically important organisms.

The taxonomic heterogeneity of this group has also reflections on the enhanced interest on species for the exploitation of commercially valuable products. The diversity of species living at the most diverse conditions offers a potential wide range of biochemical compounds and secondary metabolites, supplying substrate for the research and development of potentially interesting substances (Stengel *et al.*, 2011). Also, their potential to form large crops in otherwise unusable areas is further expanding the interests in algal related products, establishing the funding for many projects and studies worldwide (Woertz *et al.*, 2009; Pittman *et al.*, 2011; Arita *et al.*, 2015; Bhattacharjee and Siemann, 2015). Their conservation is therefore, an important focal point for the development of studies, guaranteeing the stable function of ecosystems and also the achievement of this groups' full potential exploration for biotechnology.

1.3.2 The Ecological Importance of Microalgae

Algae contribute to up to 40% of total primary production of the planet, providing the basis of the food chain for a major part of the biomass worldwide, even though some experts have stated that they represent only 0.2 % of the global primary producers' biomass (Norton *et al.*, 1996). The carbon produced by algal cells, is partially released in the environment as dissolved organic carbon, thus fueling the microbial-driven biogeochemical

cycles by providing easily available compounds, with high turn-over rates (Hotchkiss and Hall, 2014). These organisms also have a crucial role in the biogeochemical cycling of nutrients including carbon, nitrogen, phosphorous and silica (Field *et al.*, 1998; Geider *et al.*, 2001). The abundance of nutrients is highly dynamic in aquatic environments, and the limitation in nitrogen and phosphorus and can have a major influence to the primary production by the phytoplankton (Sterner and Hessen, 1994; Hessen *et al.*, 2004; Elser *et al.*, 2007), which, in turn, can help regulate the nutrient abundance in a local scale by resource consumption (Auguères and Loreau, 2015). However, the fast turnover/cell division rates (usually only a few days) of primary productivity by phytoplankton, may also be responsible for the immediate reaction of organisms to any extreme environmental change (Falkowski *et al.*, 1998), exposing their vulnerability to human influence on aquatic ecosystems, increasing the importance of establishing efficient conservation strategies.

1.3.3 Biochemical composition of microalgae and their economic potential

The large phylogenetic diversity, morphology and ecology of microalgae have a direct impact on their chemical diversity. Whilst fundamental processes such as photosynthesis, and the factors that drive it, are common to most algae, great diversity can be found in algal groups with regard to basic construction, morphological and cellular structure, and chemical composition (Stengel *et al.*, 2011). If looked from a biotechnological point of view, microalgae are unique biofactories, which combine their photosynthetic capability with the high productivity obtained by controlled microbiological cultivation (Rosenberg *et al.*, 2008). Thus, although very few microalgal species have as yet been studied for their biotechnological potential, they are already currently commercially exploited in the health, nutrition, cosmetic, aquaculture and water treatment sectors (Raja *et al.*, 2008) (Table 1.1). However, the possibilities for increased exploitation of these bioresources are wide, with applications including: material for food, fibers, fertilizers, pharmaceuticals and fuels (Ördög *et al.*, 2004; Sielaff *et al.*, 2006; Gademann and Portmann, 2008; Gantar and Svirčev, 2008; Rosenberg *et al.*, 2008; Wase and Wright, 2008).

Table 1.1 - Microalgal species currently exploited to generate commercially valuable products

Species	Taxonomic group	Main products	Application
Parietochloris incise	Trebouxiophyceae	arachidonic acid (ARA)	nutritional supplement
Haematococcus pluvialis	Chlorophyceae	asthaxanthin	nutraceuticals, cosmetics, food additives
Dunaliella sp.	Chlorophyceae	β-carotene	food additives, cosmetics, nutritional supplements
Chlorella zofingiensis	Trebouxiophyceae	astaxanthin, lutein, PUFA, lipids, protein and starch	nutraceutical, aquaculture, cosmetics
Scenedesmus spp.	Chlorophyceae	lutein, carotenoids, PUFA, lipids, protein and starch	nutritional supplement
Phaeodactylum tricornutum	diatom	EPA, fucoxanthin, biofuels	cosmetics and aquaculture
Odontella aurita	diatom	fucoxanthin, LC-PUFA, protein, oil and carbohydrate	nutraceutical
Haslea ostrearia	diatom	marinnine, fucoxanthin, LC-PUFA, protein, oil and carbohydrates	nutraceutical
Nannocloropsis spp.	Eustigmatophyceae	EPA, biofuels	cosmetics, aquaculture, biofuels
Trachydiscus minutes	Eustigmatophyceae	EPA, fuels, proteins and pigments	nutritional supplement, aquaculture, biofuels
Pavlova lutheri	Haptophyta	EPA, DHA, TAG and protein	nutraceutical and food additive
Isochrysis galbana	Haptophyta	fucoxanthin, DHA, protein, oil and carbohydrates	nutritional supplement

(adapted from (Spolaore *et al.*, 2006; Leu and Boussiba, 2014))

The potential use of algae as food supplements has been explored around the world since antiquity (Gantar and Svirčev, 2008) and today this forms the basis of multi-million dollar industry worldwide (Spolaore *et al.*, 2006; Koller *et al.*, 2014). Also, the aquaculture sector has recognized the application of microalgae as food source for larvae of aquatic organisms (Volkman *et al.*, 1989) and to enrich the nutritional value of animal feed (Spolaore *et al.*, 2006). However, these applications are restricted to a small number of species, which offer suitable characteristics, such as fast growth, cellular volume and favorable biochemical composition (Rasala *et al.*, 2014; Korczyński *et al.*, 2015).

Microalgae also have a high application potential, still mostly unexplored, in the bioremediation of wastewaters (Pacheco *et al.*, 2015), while still generating large quantities of biomass (Renuka *et al.*, 2015). They have potential to remove heavy metal pollutants, in a highly efficient and eco-friendly method, with noteworthy removal of metals even in low contaminant levels (Monteiro *et al.*, 2012; Suresh Kumar *et al.*, 2015). Their metabolically apparatus also allow nutrient capture and consequent treatment of contaminated wastewater, providing simultaneously large quantities of biomass which is suitable for utilization and exploitation for biodiesel production (Wilkie and Mulbry, 2002; Drexler *et al.*, 2014).

One of the most intensively explored biotechnological fields over the last decade has been the potential of microalgae for biofuel production, mostly due to their easy cultivation, high photosynthetic productivity, growth rates and ability to convert solar energy in biomass (Figure 1.2).

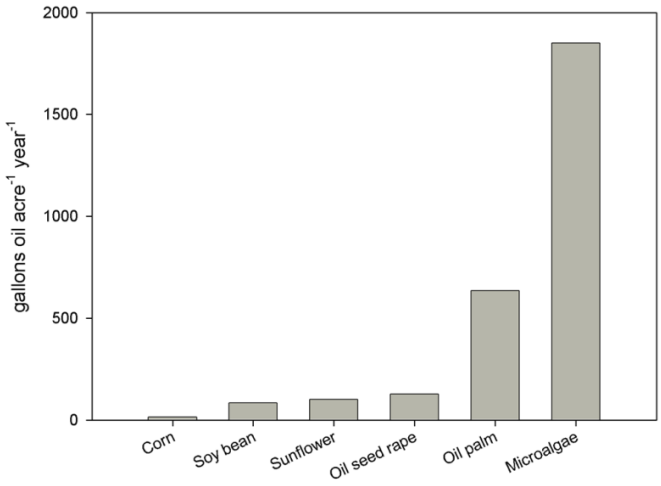

Figure 1.2 - Relative productivity levels of second & third generation biofuel candidates (http://peswiki.com/index.php/Directory:Biodiesel_from_Algae_Oil)

Microalgae also have the advantage of being relatively easily cultivable in different environments, allowing even the use of marginal lands, saline waters, wastewaters and oceans for the production of bioresources (Wilkie *et al.*, 2011). Thus, many recent studies have focused on enhancing the oil productivity (e.g. cellular content of fat acids, and their specific composition) of selected species, and promoting full searches and analysis in a wide range of organisms (e.g. Slocombe *et al*., 2015). These properties are determinant for the

establishment of economical production processes at industrial scale (Sharma and Rai, 2010).

However the limited success, some microalgae groups are also currently being cultivated and exploited for specific secondary metabolites, with high commercial value and utility in the development of health and beauty products (Forján *et al.*, 2014). The market for such materials is very large (Table 1.2) and this is an area with a lot of current research (Ganesan *et al.*, 2014; Garay *et al.*, 2014; Leu and Boussiba, 2014; Smith *et al.*, 2014; Cuellar-Bermudez *et al.*, 2015; Hlavová *et al.*, 2015), although information on the potential for freshwater microalgae is still sparse.

Table 1.2 - Existing markets for marine bio-based products

Product type and notes	Market Targets	Source	Overall estimated market
hydrocolloids agar > €150M carrageenen agarose	Food, cosmetics, pharma, research products	Macroalgae	*ca* $2bn[1]; *ca* $4.5bn[2]
Caratenoids: astaxanthin (*ca* $260m); β-carotene (*ca* $250m); lutein (*ca* $190m); canthaxanthin (*ca* $150m) - *majority synthetic*	Nutraceuticals	Microalgae	$1bn[1]
essential fatty acids and oils ('omega-3') fish oil products 75%	Nutraceuticals, foods	Microalgae	$1-1.5bn[2]
chitosan, glucosamine supplements	Nutraceuticals	crustacea	$2bn[3]
Antioxidants and other functional ingredients	cosmetics & personal care		>$700m[1]; >$20bn[2]

[1] = ingredients
[2] = direct use for food
[3] = products containing these ingredients; data from a tremendous range of sources
(modified from (Lloyd-Evans, 2010))

In addition to the eukaryotic algae, Cyanobacteria are also a potentially rich source of bioactive secondary metabolites for pharmaceutical (Moore, 1996; Namikoshi and Rinehart, 1996), or biotechnological (Belay *et al.*, 1993; Patterson and Bolis, 1997) applications.

The discovery of the potential of organisms for the production of high valued products has also interested further research in the genetic engineering for the enhancement of the efficacy of the cultivation systems, aiming the higher productivity of algal crops (Rasala *et al.*, 2014). This field also offers new possibilities for analysis and further research on different

species, with invaluable discoveries to be made, only restricted by the algae biodiversity itself.

1.3.4 Microalgal biodiversity and distribution in Brazil

Brazil is the fifth largest country in the world, occupying a territory of 8,515,692,272 km^2. The country has large hydrographic basins and 7,637 km of coastal shore. Most of its territory is under tropical climate, except for areas at the extreme south (Fig. 1.3). The 27 states are grouped in five regions: North, Northeast, Middle West, Southeast and South. According to the 2010 census (IBGE, 2010), Brazil has a population of over 190 million inhabitants. However, the country's population is not uniformly distributed, with a larger concentration in Southeast (96.92 inhabitants per km^2) and South (48.58 inhabitants per km^2). These regions are also more industrialized, and home of the main Brazilian universities and research institutions (IBGE, 2010).

It is still unknown and very difficult to estimate the precise number of algal species in Brazilian inland waters (Rocha, 2003). This is due to the difficult access for sampling, the restricted number of researchers and the insufficient infrastructure and funding required for the analysis and monitoring of natural environments, particularly on the regions North and Northeast, where most basins are still remote. However, even with the sparse available data, it is possible to note the richness of Brazilian inland waters for some groups, such as freshwater algae, where around 25% of the known world's species have been reported (Agostinho *et al.*, 2005). Currently, there are no published studies with sufficient biogeographic coverage to estimate the freshwater microalgal diversity in Brazil as a whole (Bicudo *et al.*, 1996), with only some documented regional lists of species, which do not represent the whole diversity of ecosystems in the country (Giulietti *et al.*, 2005).

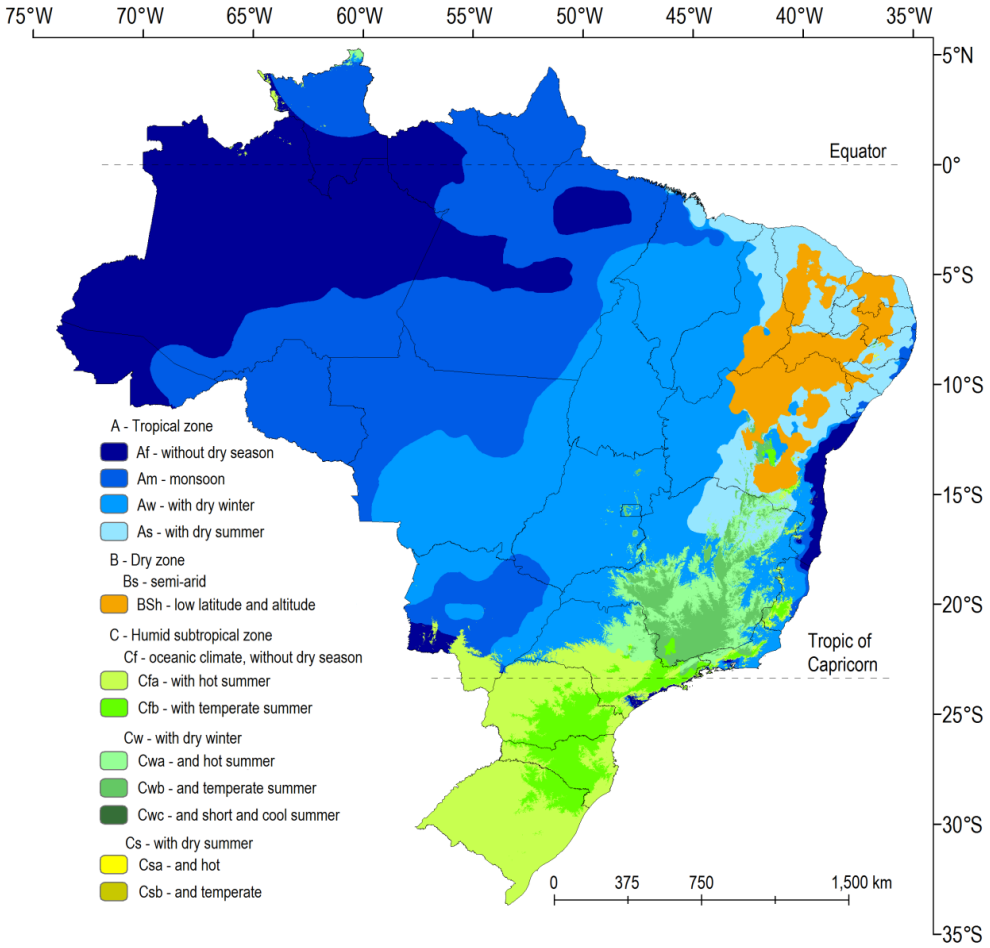

Figure1.3 - Koppen's climate classification map for Brazil

(Alvares *et al.*, 2013)

In the São Paulo State, the research group at Universidade Federal de São Carlos is working on an extensive analysis of samples from collecting points, which will cover the whole State territory. This study will be the basis of many taxonomic and biochemical papers, generating more informative species lists and will also result in recently isolated strains for further analysis. However, even such extensive work will still not be able to represent the wide diversity and potential of a country as large and diverse as Brazil.

1.4 Conservation of Biodiversity

The main objectives of the conservation of nature are the conservation of Earth's biological diversity and the safeguarding of their benefits, or 'ecosystems services' (WRI,

1999). The Earth is inhabited by several million species distributed in 100's of different phyla (Dirzo and Raven, 2003). From those projected, to date only ~1.8 million species have been described by scientists (Hilton-Taylor *et al.*, 2009), with almost 6% corresponding to freshwater species (Hawksworth and Kalin-Arroyo, 1995). However, the information available on most groups is still limited, particularly when considering the biodiversity on freshwater and inland aquatic systems (Dudgeon *et al.*, 2006), notwithstanding their importance to ecosystems function. The insufficient information available creates problems to accurately project extinction rates of aquatic organisms and, consequently limits the possibility for conservation strategies to be applied.

Most recent extinctions have been caused by human activities leading to alteration of habitats and consequently affecting their inhabitants. Thus, the conservation of biodiversity is a necessary step, even if only for selfish reasons, as to maintain the benefits the biodiversity provide, with biological resources and ecosystem services (Kearns, 2010).

Inland and freshwater biodiversity is a valuable natural resource, in economic, aesthetic, scientific and educational terms. Their conservation and management are critical to the interests of all humans, nations and governments (Dudgeon *et al.*, 2006). However, these ecosystems might be one of the most endangered in the world, with biodiversity declines far greater than the most affected terrestrial counterparts (Sala *et al.*, 2000).

1.4.1 Conservation strategies for microbial communities: *in situ* and *ex situ*

Major efforts to preserve biological diversity are underway through strategies of *ex situ* and *in situ* conservation. For microalgae, the *in situ* conservation efforts involves the creation and management of nature reserves, with the maintenance of the organisms at their source environment, continuing to interact with other biological and physical-chemical factors, avoiding the variation from the wild-type strains (Day, 1999). However, this approach is impractical for the advancement of research and knowledge-base on individual species.

For the *ex situ* approach, algal samples are conserved either as living collections or as partial material of reference (spores, DNA, etc) (Wildt *et al.*, 1997; Maunder *et al.*, 2004). The living collections usually simulate the original environment, and the strains are easily available if an organism is required quickly or if axenic cultures are needed for tests (Day *et al.*, 1999). The *ex situ* material could also be readily applied for the reproduction of threatened and endangered, as well as scientific and biotechnological exploitation (Day *et al.*, 1999; Maunder *et al.*, 2004). Culture collections may be of great importance in situations where unpredictable events may threaten rare genotypes or species in nature; when cultivation might be necessary to build numbers for reintroduction to the wild; and for genotypes who only survive *ex situ* due to total loss or alteration of their habitats (Dudgeon *et al.*, 2006; Brodie *et al.*, 2009).

1.4.2 Biological Resource Centers (BRCs) and Culture Collections

Culture collections of microorganisms can be traced to the beginning of microbiology, when microbiologists started keeping their strains alive in the laboratory as they carried out their research (Kazamia *et al.*, 2012). The first recorded service collection was the Kràl Collection established in 1890 at the German University of Prague, Czech Republic (Sly *et al.*, 1990). Currently, over 680 culture collections are listed on the WDCM database website (http://www.wfcc.info/ccinfo/), distributed in 71 countries, and maintaining 2,461,720 microorganisms, mostly bacteria and fungi. From these listed culture collections, 78 are in Brazil, holding over 100,000 microorganisms.

Culture collections differ in breadth (diversification) and depth (focus) of phylogenetic and metabolic diversity of the taxa maintained. The size of individual holdings and the expertise of curators are determined partly by financial support, partly by the history of the collections. This history also explains the respective strengths both in methods used in-house for authentication and characterization as well as in offering skills to the public by providing identification services and training courses (Stackebrandt, 2010).

When it comes to maintaining microorganisms, it is important to note that the collections have some “peculiarities”. For example, the concept of species is not as well-

defined for microorganisms as for plants and animals, making the microbial science dependant of *in vitro* organisms held in *ex situ* collections (Stromberg *et al.*, 2013).

Biological resources – living organisms, cells, genes, and the related information – are the essential raw materials for the advancement of biotechnology, human health, and research and development in the life sciences (OECD, 2007). According to OECD (OECD, 2007), Biological Resource Centres (BRCs) are *"an essential part of the infrastructure underpinning life sciences and biotechnology. They consist of service providers and repositories of the living cells, genomes of organism, and information relating to heredity and the functions of biological systems. BRCs contain collections of culturable organisms (e.g. micro-organisms, plant, animal and human cells), replicable parts of these (e.g. genomes, plasmids, viruses, cDNAs), viable, but not yet culturable organisms, cells and tissues, as well as databases containing molecular, physiological and structural information relevant to these collections and related bioinformatics".* These centers are, therefore, key components for the scientific research, industry and conservation. They can have different focus and funding, either aiming for commercial or academic purposes (Day and Stacey, 2008). They provide services dedicated to supporting a wide variety of microbiological work (Uruburu, 2003), with basic functions being maintained over the years: mechanism for *ex situ* conservation of organisms, custodians of national resources providing material to underpin the science base, repositories of strains subject to publication and to carry out safe, confidential and patent deposit services for researchers (Day and Stacey, 2008; Kazamia *et al.*, 2012).

For microorganisms, the BRCs were followed by the Global Biological Resource Centre Network demonstration project, based on the hypothesis that the full potential of microbial diversity is yet to be harnessed and a coordinated approach to resource provision will accelerate innovation and discovery (Smith *et al.*, 2014). The microbial domain Biological Resource Centre (mBRC) is available to those microbiological resource collections that implement OECD best practice, network their activities nationally and internationally for efficiency and effectiveness, meet mandatory guidance laid down in membership rules, and carry out research to add value to holdings. Many collections meet most of the criteria of a formal mBRC, although many are missing the collaborative, or networking elements, the

advanced quality control, and the adherence to international standards and regulations (Smith *et al.*, 2014).

1.4.3 Algal Culture Collections

Culture collections of microalgae represent a valuable support to scientific, educational and commercial activities throughout the world (González *et al.*, 1995; Day *et al.*, 2008). Most of the major algal culture collections (Table 1.3) were established decades ago, and work as depositories and centers for distribution of strains for national and foreign uses, for all kinds of applications: research, aquaculture, toxicity tests, educational activities, etc. These biobanks are essential for the biodiversity conservation objectives, and should focus in the expansion of their reach, playing an important role on the *ex situ* conservation of threatened algal species.

Table 1.3 - Major culture collections worldwide and number of strains held

Culture collection Acronym	Country	Number of Microalgae Strains
CCAP	UK	2500
SAG	Germany	2269
ACOI	Portugal	4000
UTEX	USA	2200
NIES	Japan	2159
NCMA	USA	2799
ANACC	Australia	1460
CCMA-UFSCar	Brazil	~700

(data from WDCM info website, retrieved on April/2015)

There are four fundamental features of collections of biological materials that must be sustained to establish the value of stored material: (I) purity; (II) authenticity (correct identification of strain and accession numbers); (III) stability; (IV) qualification data for stock

culture (Day and Stacey, 2008). The use of nonconforming biological material (e.g. misidentified microorganisms and/or contaminated cultures) can cause serious problems in research as well as in industrial processes. Well-established collections must be able to provide strains and services of high quality, and in agreement with the law and public policies (Sette *et al.*, 2013), maintaining practices to ensure that the samples of organisms they hold and distribute sustain their important characteristics.

1.4.4 Microalgae culture collections in Brazil: history and current situation

In Brazil, the first culture collection of marine microalgae was established at Instituto Oceanográfico, Universidade de São Paulo (IOUSP, located in São Paulo, SP) by Dr Clovis Teixeira and Dr Armando A. H. Vieira, in the early 1970's. In 1977, Dr Armando Vieira started the first Brazilian culture collection of freshwater microalgae at Universidade Federal de São Carlos (UFSCar, São Carlos, SP). Later, in the 1980's, other culture collections were created in Brazil and there are currently 41 protistan/algal culture collections in Brazil, the majority housed by public universities and research institutes; however, most of them maintain only a very small number of strains. Only five institutions have culture collections with more than 150 strains of microalgae: UFSCar, IBt, IOUSP, Escola Superior de agricultura Luiz de Queiroz (ESALQ/USP) and Universidade Federal Fluminense (UFF/RJ), all of them on the Southeast region of the country. Most Brazilian cultures face implementation problems, with discontinuity of activities, caused by the absence of the curator figure, showing an inability to consolidate and maintain the strains (Lourenço and Vieira, 2004).

1.5 Management of microalgae culture collections

The success of an established culture collection is directly dependant on its correct and efficient management. The first step in the correct management of a culture collection of microalgae is the correct identification of the strains held. Ideally, studies employing genetic barcoding should be performed, since the application of traditional taxonomy alone may leave doubts on the identification of some organisms (Guiry, 2012; Clerck *et al.*, 2013). It is vital that any inoculum provided by a collection to a third party is correctly identified

and in concordance with strains from different sources, guaranteeing the reproducibility of the tests applied and their products in a global scale. This is a basic requirement for their application as biological standards, use in sequential studies and biotechnological exploitation (Day *et al.*, 2005; Gachon *et al.*, 2007; Stacey and Day, 2007).

Furthermore, the maintenance methods used for the long term storage of cultures require trained personnel and aseptic techniques, to avoid the contamination of cultures, either by bacteria or even cross-contamination of unialgal cultures (Day and Brand, 2005). Any bacterial contamination, or in the case of non-axenic isolates changes in composition of the commensal bacterial flora, might alter the performance and productivity of the alga. Additionally, in a contaminated culture, the secondary metabolites produced by algae and bacteria are directly mixed, making it difficult to trace back the original producer of a determined compound (Cole *et al.*, 1982; Day *et al.*, 2012).

1.5.1 Maintenance methods in algal culture collections

Algal culture collections usually maintain their strains under controlled growth conditions (Lorenz *et al.*, 2005). These strains are available, with their associated database, to a wide range of users, including commercial and academic organizations. Thus, samples might be readily obtained, and will function as inocula, generating healthy and vigorous cultures in one to three weeks, varying with their growth rate.

1.5.1.1 Serial subculture

Serial subculturing involves transferring an inoculum from a late log/stationary phase culture into fresh, sterilized medium, applying aseptic microbiological techniques (Lorenz *et al.*, 2005). It is still the method of choice for the smaller culture collections. The main objective of this process is the maintenance of a healthy, morphologically and genetically representative population, which could be applied in tests at short notice. However, this procedure, has some limitations including the selective and artificial nature of the media and incubation regimes, which may lead to the loss of important morphological features and

physical traits (e.g. size reduction of diatom frustules (Jaworski *et al.*, 1988), pigmentation loss in several species (Warren *et al.*, 2002) and morphological alterations in Desmidiaceae (Vieira, personal communication).

Furthermore, the maintenance of metabolically active culture collections is a laborious, expensive and continuous work, requiring the presence of specialized technical support and laboratory infrastructure (Lorenz *et al.*, 2005). The laboratory infrastructure is a limiting aspect in the expansion of cultures and establishment of a culture depository, and may effectively affect the maintenance, quality and availability of the inoculums, for either scientific or commercial use (Day and Brand, 2005; Stacey and Day, 2007). Also, specialized personnel are required for the maintenance, with higher demands for bigger collections. A training period is necessary for new personnel, focused on the aseptic handling of strains in order to avoid contamination of axenic cultures, mixing or mislabeling of cultures, or any other handling mistakes (Lorenz *et al.*, 2005), which would undoubtedly lead to the deterioration and loss of inoculum, which might reduce their economical, industrial and academic value.

1.5.1.2 Freeze-drying

The process of lyophilization, or freeze-drying, removes water from the cells, stopping the metabolism and resulting in a state of dormancy in living cells (Adams, 2007). It requires limited infrastructure, and had great potential for the application for storage of microalgae for biotechnological application. However, this process had low success rates when applied to long term maintenance of algae (McLellan *et al.*, 1991; Day and Brand, 2005), with very few cultures surviving the procedure and where survival is observed levels of viability are unacceptably low (Day and Brand, 2005).

1.5.1.3 Cryopreservation

Cryopreservation is the storage of viable cells and organisms at ultra-low temperatures, usually in liquid nitrogen/ nitrogen vapor, at temperatures around -196^{o}C (Benson, 2008).

This technique has currently been applied as the most effective option for the long term storage of cultures, as it allows the living algae that do not have any normal resting stage to be maintained indefinitely in an “arrested state” (Day and Brand, 2005). Assuming the integrity of the storage tube is not compromised, the frozen collections have also reduced risk of contamination, as they requires only minimal handling (except for the periodical addition of liquid nitrogen on storage dewars and the thawing of samples). After the initial investment for the establishment of the infrastructure, frozen collections need only minor maintenance requirements, and the expansion of such collections is an easier process, providing the support for more complete sources and deposits of strains (Day and Stacey, 2007), an ever growing requirement for microalgae culture collections.

1.5.1.4 Comparison of different methods that can be employed to maintain algal cultures

The continuous maintenance of metabolically active cultures has significant disadvantages: intensive labor, the necessary resources and the error risks in manipulation, e.g. bacterial or fungi contamination. Furthermore, these cultures are continuously subjected to evolutionary changes, gene mutations, and cannot guarantee the phenotypic stability of inoculums (Jaworski *et al.*, 1988). When systematically tested drying and freeze-drying had low success rates when applied to long term maintenance of algae (McLellan *et al.*, 1991; Day and Brand, 2005), leading to the recent use of cryopreservation as the most appropriate stock method for cultures (Benson, 2008). However, cryopreservation techniques are not as yet global adopted, with many recalcitrant (i.e. not viable using standard methods) organisms, which do not survive the freezing and thawing process (Day and Brand, 2005; Day and Stacey, 2007). Thus, each methodological approach has their advantages and disadvantages (Table 1.4), which should be carefully calculated and considered in the selection of the best maintenance process for each strain, and/or groups of organisms.

Table 1.4.Comparison of maintenance methods, with advantages and disadvantages for each method

Preservation Regime	Advantages	Disadvantages
Serial subcultures	Straight forward method; Short term cost effective; Rapid culture recovery	Short time between subcultures; Risk of contamination; Genetic drift; Storage space
Freeze-Drying	Easy storage; Long storage time	Equipment; Non-suitable for most algae
Cryopreservation	Long storage time; Easily adaptable method; Reduced chance of strain deterioration; Reduced chance of contamination; Storage space	Equipment; Requires continuous supply of Liquid Nitrogen; Toxic Cryoprotective Additives (CPAs); Time of culture recovery Recalcitrant algae

(adapted from (Ryan and Smith, 2004; Day and Brand, 2005)

1.6 Cryopreservation and its applicability

Cryopreservation might be a key element required for the establishment of reference collections of microalgae, particularly in Brazil, which has extensive and rich biodiversity and few opportunities for funding and personnel training and employment, as most cultures collections are university-based. The cryobanks, once established, can reduce directly the costs and labor amounts for maintaining the culture collections, by the reduction of purchase and waste of consumables, and also the laborious risk management control (Benson, 2008). The maintenance of cultures under this regimen would improve the collections ability in providing standardized material, assuring the quality of the inoculums provided, and also increase the cost effectiveness of the laboratories. However, the establishment of a new technique requires the necessary investment on research and basic material, and focus on the development of cost-effective long-term storage techniques.

1.6.1 Basic rationale for the application of the technique

The basis for the application of cryopreservation technique is on the concept of "freezing biological time". Cryopreservation uses ultra-low temperatures 'to slow, to the point of cessation, but not death, the vital functions of the organisms' (Benson, 2008), altering their chemical kinetics and thermodynamics properties, when the energy levels

become too low to allow the necessary molecular motion (Grout *et al.*, 1990; Day and Fleck, 2015).

The freezing process consists in the reduction of samples temperature at a controlled rate, usually obtained with help of controlled rate freezers (Day and Brand, 2005). The success of the cryopreservation process in dependent on optimizing the protocol to reduce the effects of potentially injurious factors: ice and dehydration (Day and Brand, 2005). The *two factor hypothesis of freezing injury* was firstly proposed by Mazur (Mazur *et al.*, 1972), and states the importance of the chilling rate for the success of the protocol, as it determines the rate at which water moves across cell membranes, influencing the formation of intracellular ice and the cell solutes concentration (dehydration). The cooling rate should be slow enough to allow limited loss of intracellular water, reducing the risks of intracellular ice formation which could be lethal for samples, however also avoid the excessive loss of water, which could lead to plasmolysis and cell death (Mazur *et al.*, 1972). The process is usually facilitated by the addition of penetrating colligative cryoprotectants (CPA), which can prevent cell damage caused by volume changes and excessive solute concentration by depressing the freezing point of samples (Harding and Benson, 2013).

The frozen cells maintained at ultra-low temperatures (<-132^{o}C) have arrested ageing at cellular, physiological and molecular levels, and there should not be any differences on the viability of the culture, independently of the time it is stored, making it possible the maintenance of cultures for undetermined time (Mazur, 1984; Day *et al.*, 1997; Benson, 2008). The cryopreservation of living valuable strains would be beneficial, therefore, for their long term storage, reducing the risks of genetic changes and loss of commercial biosynthetic and molecular attributes (Benson, 2008).

1.6.2 Functional and genetic stability for long term storage

A cryopreserved microalgae culture is theoretically stable for how long it stays in ultra-low temperatures (Grout *et al.*, 1990). However, the process of cryopreservation *per se* has the potential to cause cryoinjury, exposing the organism to physical, chemical and physiological stress (Reed *et al.*, 2004; Harding *et al.*, 2010b). Each stage of the process has

the potential to result in irreversible damage (Fig. 1.4), so the objective in any protocol is to minimize damage to the cells to be conserved during preparatory steps the cryopreservation procedure itself and any recovery/ revival phase needed to regenerate a standard culture for subsequent use.

In addition, the extent of the cryoinjury will be dependent on a series of both physical and ecological factors (Johnston *et al.*, 2007; Johnston *et al.*, 2010), and may affect the viability of the culture, and their ability to react to natural environmental conditions. Genetic differences may also appear during the freezing-regrowth process, reflecting the importance of verifying if cryopreserved samples can be considered 'true to type' material (Day *et al.*, 2010; Harding *et al.*, 2010b).

Even though the consensus of opinion is that the process is effective (Harding *et al.*, 2010a), there have been very few studies on the phenotypic and genetic stability of cryopreserved specimens, and these have mostly been undertaken for plant cells and embryos (Fernandes *et al.*, 2008; Martín *et al.*, 2011). For microalgae, even less information can be found on undertaking a literature survey (Hédoin *et al.*, 2006; Müller *et al.*, 2007; Harding *et al.*, 2010a; Hipkin *et al.*, 2014), with large gaps on the process and possible causes of changes. As a solution to this restricted information, Harding (2007) suggested the study of "cryobionomics", involving assessing phenotypic, histological, cytological, biochemical and molecular biology knowledge, to examine the aspects of cryo-injury and how they may affect the behavior of a recovered cryopreserved sample if it were to be reintroduced in their natural habitat. This approach is scientifically sound, but has practicality and financial implications if it were to be used in a large collection.

The biochemical exploitation of strains, either wild-type or genetically modified requires the offer of ensured genotypic and phenotypic stability (Day and Stacey, 2008). Ideally, the cryopreservation method should be "robust", to offer standardized and vigorous inoculums, and fingerprinting methods should be applied prior to and after freezing the cultures, jointly with other functionality tests, to ensure the quality and applicability of a conserved strain (Benson, 2008; Day *et al.*, 2010).

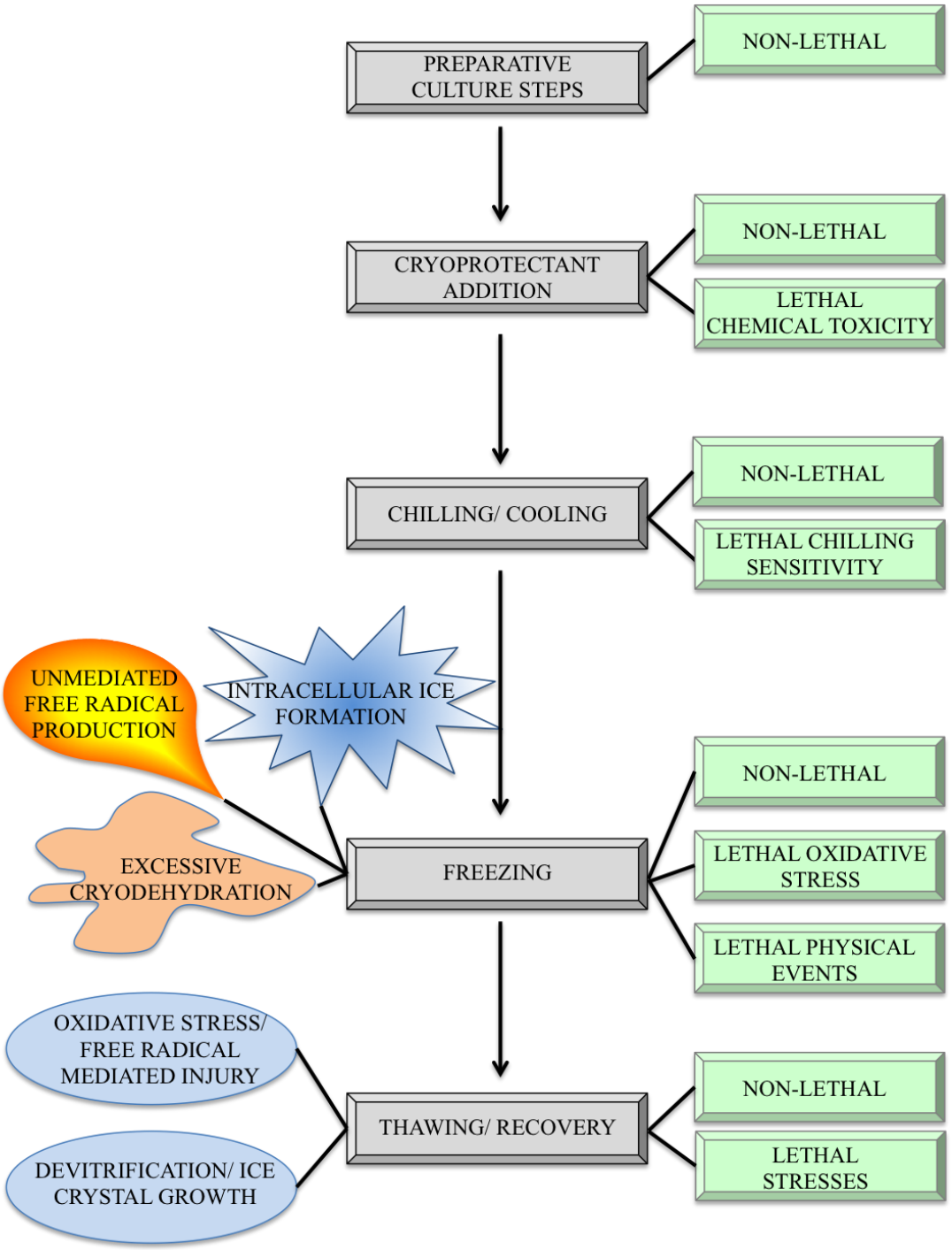

Figure 1.4 - Graphic illustration of a cryopreservation protocol and the stresses which may be associated with each step. In addition, mechanisms which contribute to injury/stress are highlighted for the freezing and thawing/recovery stages of the protocol.
(Day & Fleck, 2015)

1.7 Aims of the PhD thesis

The conservation of biodiversity is a priority focus for ecological studies currently, and actions for the *ex-situ* conservation of microorganisms are an important step on the way to guarantee the survival of many species, including organisms with potential economic value. Thus, the main objective of this study was the establishment of the cryopreserved microalgae germplasm bank, jointly with the Culture Collection of Microalgae, expanding its ability to maintain a larger number of species and in a better, cost-effective manner in comparison with metabolically active cultures.

This study was undertaken both to fulfil the above objective and to develop a body of methodologies and knowledge that would allow the subsequent development of the UFSCar collection.

The studies were focused on the establishment and application of a standardized protocol to create the basis of a cryopreserved bank, applicable to a large number of species. This additionally offered a starting point for further research on the development and optimization of specific protocols.

The analysis of the longevity of samples maintained frozen at different temperatures was also approached. This study was undertaken to test the hypothesis that maintenance of samples immersed in liquid nitrogen is important to guarantee the continuous viability of samples for long term storage. Also, a systematic study was performed at the Culture Collection of Algae and Protozoa (CCAP) in the UK to test this hypothesis, so materials that had been cryogenically stored for up to 40 years were evaluated.

The cryopreservation process *per se* is highly stressful and can cause cell damage. Work was undertaken to assess genotypic and functional stability of samples during the cryopreservation process. Cryopreserved microalgae were evaluated therough physiological tests and genetic analysis, to explore the hypothesis that cryopreservation does not alter significantly the organisms characteristics.

The recovery of a cryopreserved axenic strain is dependent on the cells ability to capture nutrients and reproduce. However, the recovery process can be affected by the presence of

a bacterial community. The relationship between the algal population and bacterial community after cryopreservation was studied, with the objective of assessing the effect on the viability of the microalgae culture during the process.

Finally, the successful establishment and expansion of a biobank, or cryobank, is dependent on the development and management of processes and quality control protocols for the deposited samples. Thus, based on a systematic Quality Assessment (Q/A) exercise undertaken during a BEPE funded placement at the Culture Collection of Algae and Protozoa (CCAP) in the UK, the pathway for the development of procedures applicable in the Culture Collection of Microalgae, at UFSCar (Brazil), was explored.

In addition to the core work undertaken as part of this PhD study, information obtained was compiled on a culture collection database and incorporated into the more global BIOTA project database, helping to get a better understanding of the algal biodiversity of São Paulo State.

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2 Establishment of a cryopreserved biobank for the UFSCar Culture Collection of Freshwater Microalgae (CCMA-UFSCar), São Paulo, Brazil

2.1 Abstract

The Culture Collection of Freshwater Microalgae (CCMA-UFSCar, Coleção de Culturas de Microalgas de Água-Doce), based at the Universidade Federal de São Carlos, plays an important role in underpinning Brazilian microalgal research, providing biological materials, substrates and training personnel for a large proportion of the past and current projects in this area. However, recent efforts to expand the culture catalogue/holdings have reached a ceiling because of the logistical practicality of maintaining actively growing cultures. To reduce the costs associated with the maintenance regime of cultures, efforts were initiated on the establishment of a cryopreserved biobank for long term maintenance of cultures, thus minimizing the efforts associated with handling of material, as successfully frozen cultures, in theory, could be maintained effectively indefinitely, with the advantage of the stability of cells characteristics. Initial tests for freezing samples with a standard protocol were performed on 93 strains including taxa across the different taxonomic groups in the collection catalogue, accessing the post-thaw viability of samples. The highest levels of success were achieved for the smaller taxa, such as small green algae; while for the larger and more complex organisms limited to no success was obtained. For the strains tested, over 70% had adequate levels of post-thaw viability and regenerated phenotypically robust cultures.

Keywords: Cryopreservation; freshwater microalgae; biobank.

2.2 Introduction

The Culture Collection of Freshwater Microalgae (Coleção de Culturas de Microalgas de Água-doce – CCMA-UFSCar), based at Universidade Federal de São Carlos, was the first of its genre to be established in Brazil, in 1977, by Prof. Dr. Armando A. H. Vieira (Lourenço and

Vieira, 2004). It is currently the largest freshwater microalgae collection in Brazil, holding around 700 strains of freshwater microalgae, mostly isolated from diverse water bodies in Sao Paulo State, Brazil. This collection has been the source of samples for research undertaken by both public and private institutions, across a wide range of academic and biotechnological projects. Furthermore, it has also been responsible for the specialized training of personnel, including researchers which went to initiate other algae culture collections in Brazil. Recently, renovated efforts were concentrated in the expansion of the collections holdings, as part of a thematic project (Processo FAPESP 2011/50054-4) connected to the BIOTA-Fapesp network. This Program was focused on the discovery, mapping and analysis of the flora and fauna of the State of São Paulo, and also the evaluation of sustainable exploitation of plants or animals with economic potential which will assist in the formulation of conservation policies on forest remnants (FAPESP, 2008).

Traditionally, strains at CCMA-UFSCar were maintained in replicate metabolically active cultures under suboptimal conditions (Vieira, pers. comm), which must be sub-cultured every 2-3 months to maintain their viability. These cultures are readily available for further application and distribution; however, for their successful maintenance, conditions must be carefully considered and met, such as the culture medium, light, temperature and the intervals between transfers (Lorenz *et al.*, 2005). Also, species may greatly differ in their needs for growth, requiring a good designed space and trained personnel for handling, raising the investment necessary for the upholding of the collection (Brand *et al.*, 2013). Also, the sub-culturing carries several risks for the cultures, mostly related to the transferring activities, as it opens possibilities for cross-contamination of samples, mislabeling, or contaminations from outside organisms, which can lead to the loss of strains when not carefully monitored (Day and Brand, 2005; Lorenz *et al.*, 2005; Brand *et al.*, 2013) or raise the requirements for their maintenance with the re-isolation and decontamination over time. The high costs of maintenance also reduces the opportunities for the expansion of the collection holdings, which has become a necessity after the ever-expanding growth on interest in these organisms.

In order to reduce the costs of maintenance and the exposure of strains to sources of contamination and deterioration of samples, cryopreservation is recommended for the long

term maintenance of samples in an arrested state (Day, 2007). This type of methodology reduces the routine costs for the culture collection, increasing the capacity of expansion of the holdings while insuring the stability and purity of samples and reducing the risks of contamination during the handling of samples. The recent expansion of the CCMA-UFSCar holdings is raising the volume of work for the maintenance of active cultures, which is rapidly reaching unsustainable levels under the current regime in the terms of investment and resource requirements. Furthermore, recently, contamination events by fungus and other organisms have occurred, which in turn increased the workload associate with the collections curation. Therefore, efforts were raised and focused on the establishment of a cryopreserved biobank for the culture collection, in order to secure the collection holdings, which are the focus of much of the research work of the laboratory and source of samples for many research groups in Brazil.

2.3 Material and Methods

2.3.1 Establishment of the cryobank infrastructure

The CCMA-UFSCar has been established as a culture collection for almost 40 years at its current location, maintaining metabolically active cultures. It is registered at the World database for Culture Collections with the reference WDCM 835. However, cryopreservation efforts were only recently initiated. Thus, adjustments of the laboratory infrastructure were necessary to facilitate the successful implementation of cryopreservation protocols for the maintenance of existing and recently incorporated microalgae strains from the culture collection. The acquisition of the necessary equipment was funded by the FAPESP Thematic Project (2011/50054-4), and included (Fig. 2.1): a controlled rate freezer (CRF) Planer Kryo 360 1.7 (Planer PLC, UK) with associated software which allows the control of the cryopreservation process; an Ultra-Low temperature freezer (Revco Value Series, Thermo Scientific, USA); sample storage dewar SC-47 R (Sempercrio, Brazil) and nitrogen transportation dewar SC-60/I (Sempercrio, Brazil).

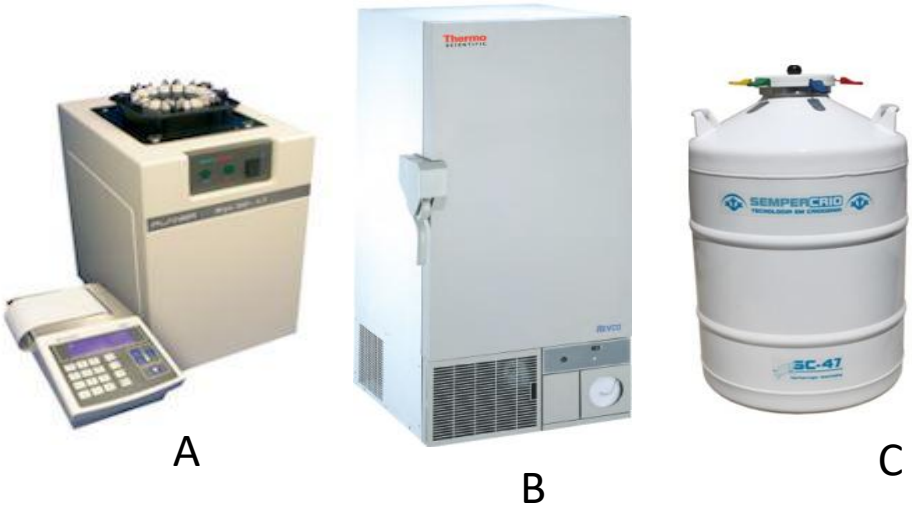

Figure 2.1 - Equipment incorporated to the Phycology Laboratory (Universidade Federal de São Carlos) facilities to assist the incorporation of cryopreservation protocols to the routine maintenance of cultures: A) controlled rate freezer (CRF) Planer Kryo 360 1.7 (Planer PLC, UK); B) Ultra-Low temperature freezer; C) sample storage dewar.

2.3.2 Training of Personnel and development of protocols

As part of the initial activities of the project, the training on the required techniques was underwent at the Culture Collection of Algae and Protozoa (CCAP), based at the Scottish Association for Marine Science (SAMS), Oban, UK, under Prof. Dr. John Day supervision, in February 2012. The CCAP has recognized work and over 40 years expertise on the maintenance of frozen strains of marine and freshwater algae and protozoa. The training included basic theory and practical protocols for the successful maintenance of strains at ultra-low temperatures, which were further adapted for applicability using the Brazilian laboratory facilities. These experiences were applied for the development of the standard cryopreservation procedure for conservation of freshwater strains at CCMA-UFSCar.

2.3.3 Collection Holdings

CCMA-UFSCar currently holds almost 700 strains of freshwater microalgae, from a wide range of groups (Table 2.1). From these, 133 strains are currently maintained in axenic

cultures, which are periodically checked with WC medium (Guilard and Lorenzen, 1972) modified by the addition of glucose and peptone (250 $mg.L^{-1}$ each). The remaining cultures of the collection are maintained as uni-algal cultures after isolation from environmental samples.

Table 2.1 Distribution of the current collection holdings of the CCMA-UFSCar on major taxonomic groups.

Taxonomic group	no. of strains
Cyanobacteria	**65**
Chlorophyta	
Chlorophyceae	273
Trebouxiophyceae	68
Ulvophyceae	5
Unidentified	4
Charophyta	
Conjugatophyceae	110
Klebsormidiophyceae	1
Cryptophyta	
Cryptophyceae	5
Dinophyta	
Dinophyceae	2
Euglenophyta	
Euglenophyceae	2
Heterokontophyta	
Bacillariophyceae	43
Chrysophyceae	3
Coscinodiscophyceae	18
Raphidophyceae	1
Synurophyceae	7
Xanthophyceae	2
Unidentified strains	**76**
	685

For the initial establishment of the cryopreserved biobank, efforts were focused on the maintenance of the axenic strains, which are currently the main focus of further research at the Phycology Laboratory at UFSCar. The tests included 93 strains of freshwater microalgae, mostly Chlorophyceae (62 strains), but also covering organisms from

Conjugatophyceae (11 strains), Trebouxiophyceae (7 strains), Cryptophyceae (1 strain), Synurophyceae (1 strain), Xantophyceae (2 strains) and Bacillariophyceae (9 strains).

2.3.4 Freezing Protocol

Initially, a commonly employed protocol was applied for all the tested organisms, to establish the main database for the maintenance of cryopreserved organisms at CCMA-UFSCar. For this, cryoprotectant solutions of dimethyl sulfoxide (DMSO) were prepared previously in culture medium at 10% concentration (v/v). All strains of microalgae were cryopreserved using a two-step, controlled rate cooling protocol (Day and Brand, 2005). Briefly, an aliquot of 0.5 mL 10% (v/v) cryoprotectant solution was added to 0.5 mL of a dense microalgal culture in a cryovial, which was incubated for 15 min at room temperature and out of direct light to ensure the cryoprotectant action. Samples were then transferred to a controlled rate cooler (Planer, UK) at a start temperature of 20 ^{o}C. Cultures were then frozen at a cooling rate of -1^{o}C.min^{-1} to -40 ^{o}C, held at this temperature for 15 min and then plunged into liquid nitrogen. Samples were transferred to local cryostorage facility and three samples of each cryopreserved strain were thawed for viability analysis.

2.3.4.1 Toxicity of the CPA solutions

Simultaneously to the freezing protocols, the toxicity of DMSO 5% (final concentration during cryopreservation) was tested for several strains of microalgae. This test consisted in the mixture equal parts of CPA solution (10%) and microalgae culture, which were incubated for 15 min at room temperature. Aliquots of 1 mL of the samples were then diluted in 10 mL of fresh medium, and maintained to the regular culturing conditions of the bank: 100 µmol m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark:light cycle and at a temperature of 23 ± 1^{o}C for growth. The viability of these samples was determined after 2-3 weeks of growth, similar to frozen samples.

3.4.2 Alternate freezing protocols: analyzing the success rates of dimethylsulfoxide (DMSO) and methanol (MeOH) for the cryoprotection of samples.

The possibility of application of MeOH as an alternative to DMSO for the cryopreservation of 18 strains of microalgae was verified. In this test, the cooling protocol previously described was maintained, exchanging the CPA solution from DMSO 10% for a methanol 10% solution, prepared under the same conditions. For this test, viability measurements were performed using vital staining, with erythrosine-b.

2.3.4.3 Cryopreservation of **Chlamydomonas chlorastera***: assessment of different cooling rates and cryoprotectant agents.*

The initial cryopreservation of *Chlamydomonas chlorastera* (CCMA-UFSCar 009) with the previously described two-step protocol using DMSO 5% as CPA had no success on the recovery of samples. Thus, several adaptations on the cooling rates and CPA employed for cell protection during the process were verified in order to obtain the most successful protocol for the maintenance of this species in liquid nitrogen for long term storage. For this, combinations between three different CPAs (methanol, DMSO and Glycerol) and four different cooling rates were tested, generating 20 different freezing treatments (Table 2.5). The toxicity of the CPA in the samples was also assessed by the exposure of samples to similar to freezing concentrations of these compounds, before dilution to innocuous concentrations of these compounds. The viability of samples was assessed using the absorbance of samples after recovery and subsequent growth.

2.3.5 Thawing of samples and Viability analysis

The stored cryovials were transferred from the cryostore to a small Dewar containing liquid nitrogen. These were thawed in a pre-heated water bath (40^{o}C) until all visible ice had melted (Day and Stacey, 2007). They were then rapidly transferred to a laminar flow cabinet, the outside of the cryovial wiped with 70% (v/v) ethanol, caps removed and using a sterile

pipette, the contents (0.5 ml) from each of the three thawed replicate samples were transferred into culture tubes with 10 mL of WC medium to dilute the potentially toxic characteristics of the cryoprotectant. Cultures were maintained for 24-36h in the dark and then exposed to the regular culturing conditions of the bank: 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark:light cycle and at a temperature of 23 ± 1^{o}C.

Assessment of viability was performed 2 - 3 weeks of incubation under the above cultivation regime, with analysis of the optical density and the fluorescence of Chl-a using spectrophotometer (HACH) and Trilogy Laboratory Fluoremeter (Turner Designs, USA) equipped with a chlorophyll *in vivo* Module (7200-043), respectively. The viability of samples after freezing and storage was assessed indirectly, with analysis of the growth rate of the cultures obtained after recovery of samples. For this, the raw fluorescence of chlorophyll-*a* was quantified on triplicate initial cultures (immediately after inoculation) and, after 2 - 3 weeks, in the recovery culture tubes of each treatment and control cultures. For comparisons between treatments, growth rate for cultures was calculated according with the equation (Wood *et al.*, 2005):

$$r = \frac{\ln (N_t/N_0)}{\Delta t}$$

where: N_0 is the initial raw fluorescence of cultures when assembled (RFU), N_t is the final fluorescence of each replicate (RFU) and Δt was the time measured in days.

Alternatively, for the comparison between the cryopreservation success using DSMO and MeOH as CPA, the viability of samples was assessed using microscopical analysis of samples stained with erythrosine-b saturated solution (SIGMA) (Calomeni and Rodgers, 2015). This stain can only permeate dead cells, which will acquire the red color, while the living (viable) cells maintain their natural green color. The viability of samples before cryopreservation was assessed for control and standardization of results. After cryopreservation, 100 uL of the culture were separated in a 200 uL PCR tube, to which 30 uL of a saturated erythrosine solution was added and mixed. After 15 min, samples were analyzed in optical microscopy and the number of cells stained (red) and not stained (green)

annotated, to a minimum of 50 cells counted in each slide. The viability of the samples was calculated according with the formulae:

$$Viability\ (\%) = \frac{n.of\ living\ cells\ (green)}{Total\ cells\ counted\ (green + red)} x100$$

In order to avoid the differences of viability natural for the cultures, the viability of the original cultures (prior to freezing) was considered:

$$Viability\ (\%) = \frac{Viability\ sample}{Viability\ original\ culture} x100$$

2.3.6 Statistics

When applicable, comparisons between treatments with DMSO and MeOH were performed using t-tests to determine the species in which differences of viability were observed.

2.4 Results

The applicability of the classic two-step protocol tested in this project generated a range of results, considering the wide range of groups in the 93 strains analyzed. The distribution of these results is summarized in Figure 2.2.

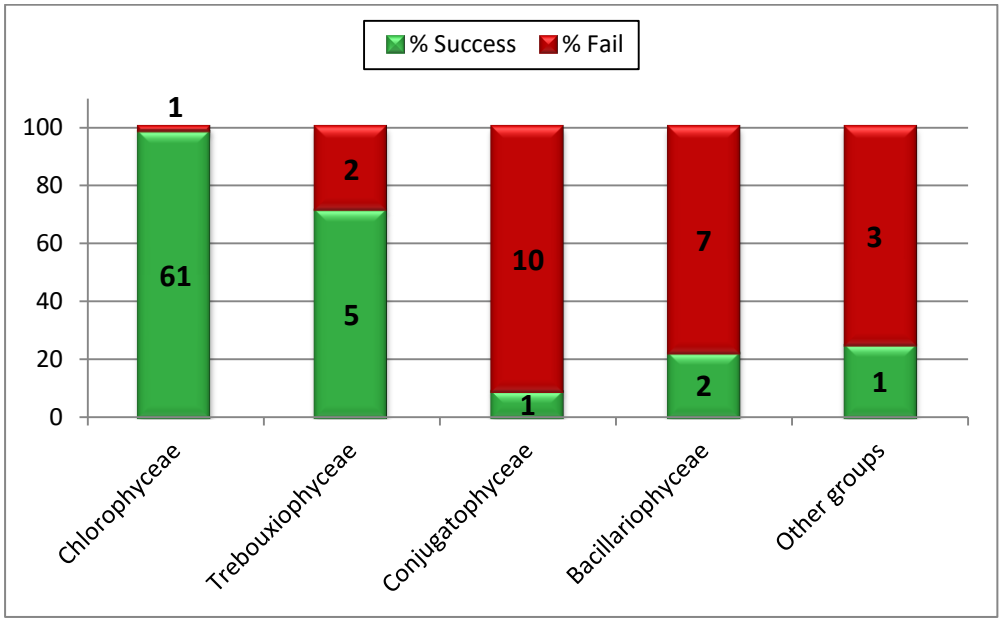

Figure 2.2 - Success and failure percentages for the cryopreservation protocol applied for different groups of freshwater microalgae. The numbers inside the bars are the number of strains tested in each taxonomic group.

The group, with the highest success rates of recovery after freezing was the Chlorophyceae, with 61 strains successfully maintained (98%). The application of the standard method to the Conjugatophyceae and diatoms was the least successful (9% and 22%, respectively).

For the Chlorophyceae, the family Selenastraceae was the most tested group, with 44 strains (Table 2.2). Most species of this group successfully recovered after cryopreservation; however, a few exceptions were observed with strains of *Selenastrum bibraianum* and two strains of *Kirchneriella* (Table 2.2).

Table 2.2 - Viability of samples from the family Selenastraceae (Chlorophyceae) after recovery from cryopreservation protocol.

Accession number		Strain	CPA	r ABS (%)	
CCMA-UFSCar	003	*Ankistrodesmus densus*	DMSO 5%	81	✓
CCMA-UFSCar	005	*Messastrum gracile*	DMSO 5%	89	✓
CCMA-UFSCar	024	*Monoraphidium arcuatum*	DMSO 5%	95	✓
CCMA-UFSCar	047	*Selenastrum bibraianum*	DMSO 5%	52	✓
CCMA-UFSCar	048	*Raphidocelis subcapitata*	DMSO 5%	47	✓
CCMA-UFSCar	063	*Selenastrum bibraianum*	DMSO 5%	7	✗
CCMA-UFSCar	083	*Ankistrodesmus flexuosus*	DMSO 5%	73	✓
CCMA-UFSCar	123	*Kirchneriella aperta*	DMSO 5%	33	✓
CCMA-UFSCar	125	*Selenastrum bibraianum*	DMSO 5%	55	✓
CCMA-UFSCar	128	*Ankistrodesmus densus*	DMSO 5%	100	✓
CCMA-UFSCar	137	*Chlorolobion cf. braunii*	DMSO 5%	89	✓
CCMA-UFSCar	168	*Selenastrum bibraianum*	DMSO 5%	76	✓
CCMA-UFSCar	174	*Kirchneriella irregularis var. spiralis*	DMSO 5%	85	✓
CCMA-UFSCar	176	*Monoraphidium griffithii*	DMSO 5%	100	✓
CCMA-UFSCar	230	*Kirchneriella irregularis*	DMSO 5%	84	✓
CCMA-UFSCar	234	*Kirchneriella irregularis var. spiralis*	DMSO 5%	100	✓
CCMA-UFSCar	239	*Ankistrodesmus densus*	DMSO 5%	100	✓
CCMA-UFSCar	241	*Selenastrum bibraianum*	DMSO 5%	62	✓
CCMA-UFSCar	278	*Ankistrodesmus stipitatus*	DMSO 5%	95	✓
CCMA-UFSCar	306	*Monoraphidium contortum*	DMSO 5%	100	✓
CCMA-UFSCar	325	*Monoraphidium pseudobraunii*	DMSO 5%	61	✓
CCMA-UFSCar	333	*Monoraphidium kormakovae*	DMSO 5%	100	✓
CCMA-UFSCar	345	*Kirchneriella obesa*	DMSO 5%	75	✓
CCMA-UFSCar	346	*Kirchneriella pseudoaperta*	DMSO 5%	63	✓
CCMA-UFSCar	348	*Kirchneriella irregularis*	DMSO 5%	94	✓
CCMA-UFSCar	349	*Monoraphidium contortum*	DMSO 5%	100	✓
CCMA-UFSCar	350	*Curvastrum pantanale*	DMSO 5%	92	✓
CCMA-UFSCar	353	*Monoraphidium kormakovae*	DMSO 5%	90	✓
CCMA-UFSCar	423	*Ankistrodesmus bernardii*	DMSO 5%	82	✓
CCMA-UFSCar	447	*Kirchneriella contorta var. elegans*	DMSO 5%	74	✓
CCMA-UFSCar	455	*Chlorolobion braunii*	DMSO 5%	100	✓
CCMA-UFSCar	462	*Chlorolobion braunii*	DMSO 5%	92	✓
CCMA-UFSCar	470	*Messastrum gracile*	DMSO 5%	94	✓
CCMA-UFSCar	476	*Chlorolobion braunii*	DMSO 5%	100	✓
CCMA-UFSCar	478	*Raphidocelis subcaptata*	DMSO 5%	89	✓
CCMA-UFSCar	492	*Pseudokirchneriella elongata*	DMSO 5%	100	✓
CCMA-UFSCar	498	*Pseudokirchneriella elongata*	DMSO 5%	81	✓
CCMA-UFSCar	516	*Kirchneriella obesa*	DMSO 5%	93	✓
CCMA-UFSCar	549	*Monoraphidium indicum*	DMSO 5%	100	✓

(table 2.2 cont.)

Accession number		Strain	CPA	r ABS (%)	
CCMA-UFSCar	604	*Chlorolobion lunatum*	DMSO 5%	100	✓
CCMA-UFSCar	606	*Monoraphidium irregularis*	DMSO 5%	79	✓
CCMA-UFSCar	609	*Desmodesmus comunis*	DMSO 5%	96	✓
CCMA-UFSCar	611	*Ankistrodesmus fusiformis*	DMSO 5%	93	✓
CCMA-UFSCar	622	*Messastrum gracile*	DMSO 5%	100	✓

(rABS: The analysis was based on the growth rate calculated using the optical density of samples at two different points in time, compared with the control cultures (non-cryopreserved). The colors indicate the relative success of growth: green – good growth, yellow – medium success and red – poor growth.)

As observed for most of the strains of the family Selenastraceae, the species tested of the family Scenedesmaceae had also high viability levels after application of the standard cryopreservation protocol, and all the samples tested were able to grow robust cultures on transfer to fresh medium (Table 2.3).

Table 2.3 - Viability of samples from the family Scenedesmaceae (Chlorophyceae) after recovery from cryopreservation protocol.

Accession number		Strain	CPA	r ABS (%)	
CCMA-UFSCar	029	*Scenedesmus bijugus*	DMSO 5%	80	✓
CCMA-UFSCar	030	*Desmodesmus comunis*	DMSO 5%	86	✓
CCMA-UFSCar	046	*Desmodesmus spinosus*	DMSO 5%	75	✓
CCMA-UFSCar	060	*Coelastrum sphaericum*	DMSO 5%	78	✓
CCMA-UFSCar	062	*Desmodesmus spinosus*	DMSO 5%	88	✓
CCMA-UFSCar	088	*Scenedesmus ecornis*	DMSO 5%	82	✓
CCMA-UFSCar	244	*Verrucodesmus verrucosus*	DMSO 5%	79	✓
CCMA-UFSCar	326	*Hariotina reticulata*	DMSO 5%	100	✓
CCMA-UFSCar	493	*Hariotina reticulata*	DMSO 5%	81	✓
CCMA-UFSCar	609	*Desmodesmus comunis*	DMSO 5%	96	✓

(rABS: The analysis was based on the growth rate calculated using the optical density of samples at two different points in time, compared with the control cultures (non-cryopreserved). The colors indicate the relative success of growth: green – good growth, yellow – medium success and red – poor growth.)

The potential toxicity of DMSO at 5% as a CPA was not a determinant factor for the cryopreservation of most microalgae strains tested. Measurements of optical density of samples after 2-3 weeks of growth showed similar patterns for samples with and without treatment with DMSO for 15 min, before dilution in fresh medium (Fig. 2.3).

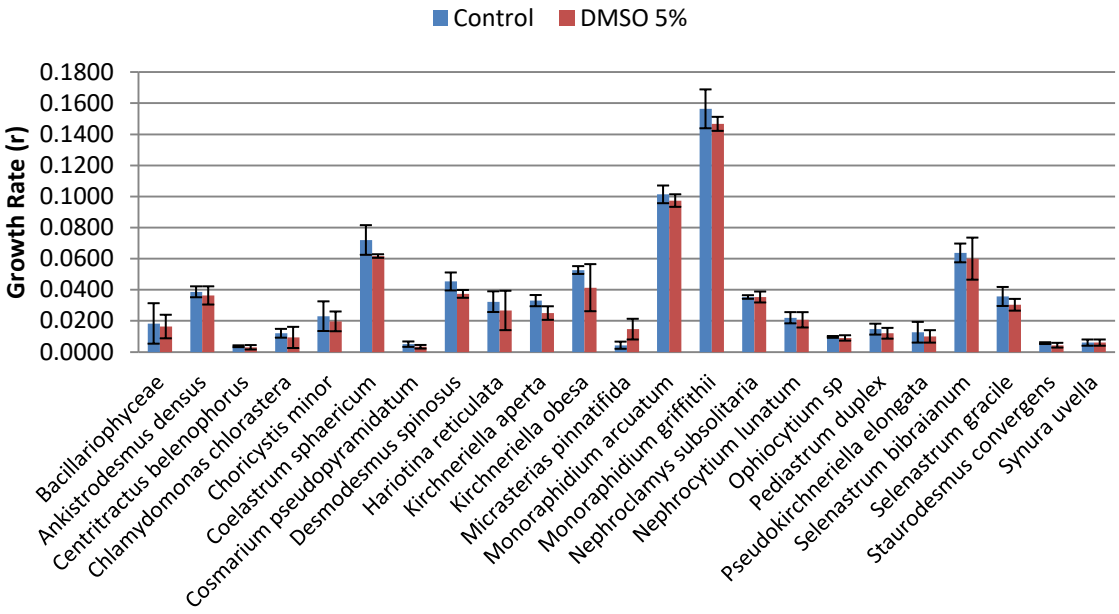

Figure 2.3 - Comparison of growth (Optical Density) between samples untreated (Control) and treated (DMSO 5%) with CPA and incubated for 15 min before dilution in fresh medium. The cultures were grown for 2-3 weeks.

For most of the strains tested (15 strains), no differences were observed in the results of cryopreservation using DMSO or MeOH as CPA (Fig. 2.4). However, for *K. obesa*, *K. lunaris* and *P. duplex,* cryopreservation with methanol had slightly better levels of success, although both treatments were able to form new robust cultures in 2 weeks growth.

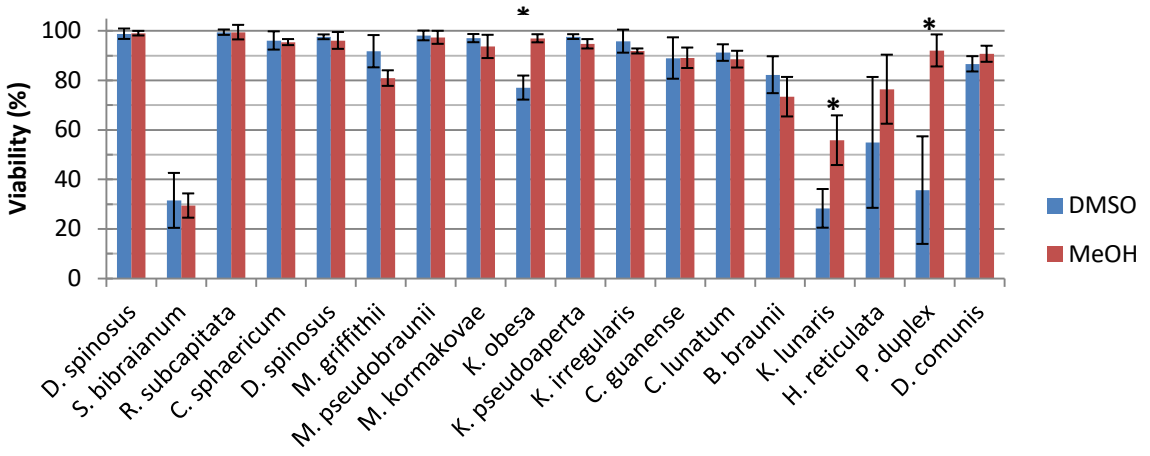

Figure 2.4 - Viability (%) of samples cryopreserved using different CPAs (DMSO and MeOH) for protection of the cultures.
*** = statistically significant differences in recovery after cryopreservation with MeOH or DMSO ($p<0.05$).**

Only one of the desmid strains (Conjugatophyceae) tested demonstrated successful regrowth after the cryopreservation protocol was completed: *Spondylosium pygmaeum* (CCMA-UFSCar 014). The remaining 10 strains tested of this group had null viability, i.e. they were not able to form new cultures (Table 2.4).

Table 2.4 - Viability of samples of desmids (Conjugatophyceae) after recovery from cryopreservation protocol.

Accession Number	Strain	CPA	r Fluor (%)	
CCMA-UFSCar 014	*Spondylosium pygmaeum*	DMSO 5%	85	✓
CCMA-UFSCar 016	*Cosmarium pseudopyramidatum*	DMSO 5%	0	✗
CCMA-UFSCar 020	*Hyalotheca dissiliens*	DMSO 5%	0	✗
CCMA-UFSCar 028	*Pleuratenium ovatum*	DMSO 5%	0	✗
CCMA-UFSCar 069	*Pleurotaenium trabecula*	DMSO 5%	0	✗
CCMA-UFSCar 076	*Staurodesmus convergens*	DMSO 5%	0	✗
CCMA-UFSCar 078	*Cosmarium pseudopyramidatum*	DMSO 5%	0	✗
CCMA-UFSCar 084	*Euastrum evolutum*	DMSO 5%	0	✗
CCMA-UFSCar 086	*Staurastrum tohopekaligiensis*	DMSO 5%	0	✗
CCMA-UFSCar 089	*Micrasterias pinnatifida*	DMSO 5%	0	✗
CCMA-UFSCar 126	*Spondylosium panduriforme*	DMSO 5%	0	✗

The analysis was based on the growth rate calculated using the Chl-a fluorescence of samples at two different points in time, compared with the control cultures (non-cryopreserved). The colors indicate the relative success of growth: green – good growth, yellow – medium success and red – poor growth.

The experiments with diatoms also had limited success, as only 2 of the 9 strains tested were able to regrow after undergoing the cryopreservation protocol (Fig. 2.5).

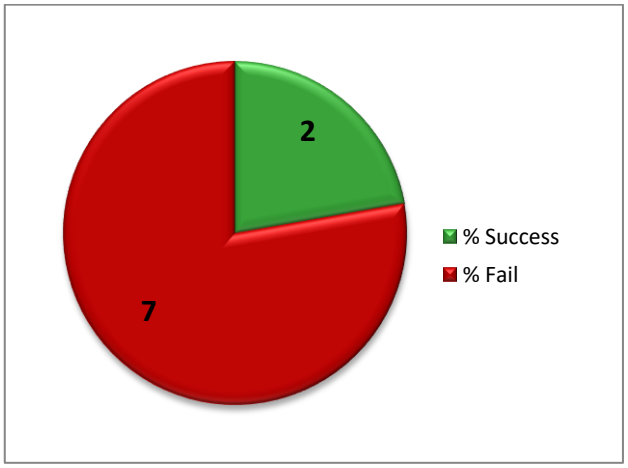

Figure 2.5 - Success and failure percentages for the cryopreservation protocol applied for Bacillariophyceae strains. The numbers inside the pieces are the real number of strains in each group.

For the study on cryopreservation of *Chlamydomonas chlorastera* a total of 20 different combinations of CPA and cooling rates were tested. The objective was to maximize viability levels, as demonstrated by the ability to re-grow robust cultures that were comparable to control cultures (i.e. not frozen sample(s)). MeOH was found to be an effective CPA, capable of protecting the cells from the freezing damage (Table 3). However, the use of DMSO or Glycerol as CPAs was not effective for the protection of cultures during the process and no re-establishment of cultures was observed for these samples. It was noted that, cultures that were exposed to the CPA for a short period of time before dilution and subsequent incubation under standard conditions showed no inhibitory effects that could be attributed to these compounds at the concentrations tested. This led to the conclusion that the damage happened during the freezing/thawing of cultures and that these compounds had little or no colligative cryoprotective effect for this alga under the cooling regime employed.

Table 2.5 - Viability of samples *Chlamydomonas chlorastera* (CCMA-UFSCar 009) after recovery from different cryopreservation protocols, using DMSO, MeOH or Glycerol as CPA and frozen at three different cooling rates.

Treatment	CPA	Cooling rate	
T01	no	not frozen	✓
T02	no	LN	×
T03	no	-1 ^{o}C min^{-1}	×
T04	no	-5 ^{o}C min^{-1}	×
T05	no	-0,5 ^{o}C min^{-1}	×
T06	DMSO 5%	not frozen	✓
T07	DMSO 5%	LN	×
T08	DMSO 5%	-1 ^{o}C min^{-1}	×
T09	DMSO 5%	-5 ^{o}C min^{-1}	×
T10	DMSO 5%	-0,5 ^{o}C min^{-1}	×
T11	MeOH 5%	not frozen	✓
T12	MeOH 5%	LN	×
T13	MeOH 5%	-1 ^{o}C min^{-1}	✓
T14	MeOH 5%	-5 ^{o}C min^{-1}	✓
T15	MeOH 5%	-0,5 ^{o}C min^{-1}	✓
T16	Glycerol 5%	not frozen	✓
T17	Glycerol 5%	LN	×
T18	Glycerol 5%	-1 ^{o}C min^{-1}	×
T19	Glycerol 5%	-5 ^{o}C min^{-1}	×
T20	Glycerol 5%	-0,5 ^{o}C min^{-1}	×

(The colors indicate the relative success of growth after visual analysis: ✓ – good growth and × – no growth.)

2.5 Discussion

2.5.1 CPA: toxicity x protective effect

The cryopreservation process results in a range of complex physical, biophysical and chemical changes in both the cells being cryopreserved and their immediate environment, thus careful consideration is required in order to achieve the highest post-thaw viability for samples (Day and Fleck, 2015). One critical point for the success of the process is the addition of a cryoprotective additive (CPA), which is almost always necessary for organisms to survive the lethal consequences of the freezing/thawing process (Doebbler, 1966). Penetrative CPAs, such as MeOH and DMSO can rapidly permeate cell membranes, which in addition to their colligative capabilities of keeping salts in solution, affect the ice crystal nucleation by forming strong hydrogen bonds with water molecules (Yu and Quinn, 1994;

Weng *et al.*, 2011). However, altering the solute composition and concentration might have extremely toxic effects and cause inhibition of the growth of cultures (Yu and Quinn, 1994; Hubalek, 2003).

The basic two-step cryopreservation protocol tested for the CCMA-UFSCar microalgae required the exposure of cultures for a minimum 15 min to DMSO 5% concentrations. As observed for the growth of cultures treated with CPAs (CPA control cultures), for most samples no inhibitory effects were observed from this step and cultures maintained their culture formation ability. Indeed, this approach adheres to this recommended use of DMSO concentrations lower than 15% to avoid the effects on microalgae cultures, and to the considerations on the temperature of reaction and time of exposure (Hubalek, 2003).

2.5.2 Cryopreservation of Chlorophyceae

Over 50% of the CCMA-UFSCar biobank holdings are composed of strains belonging to the Phylum Chlorophyta, of which almost 80% are Chlorophyceae strains, which can be further divided into 20 families. These organisms are the focus of the most recent publications from the research group (de Moraes and Vieira, 2014; Vieira *et al.*, 2016; Garcia *et al.*, 2017), more specifically taxa belonging to the order Sphaeropleales and its families Selenastraceae and Scenedesmaceae. These groups are composed mostly of small organisms, which can be unicellular, or in many cases form small colonies, and thus present a wide range of phenotypic forms (Garcia *et al.*, 2017). Successful cryopreservation of organisms from these groups was verified employing the classic cryopreservation protocol in this study (Fig 2.2; Tables 2.2 and 2.3).

Organisms with reduced sized and morphological simplicity are generally expected to be the most tolerant to the cryopreservation process (Day and Brand, 2005). The success of the freezing/thawing process is entirely dependent on the equilibrium of water loss by the cells, during the cooling process to avoid the formation of intracellular ice, while maintain the necessary conditions for cell survival (Mazur, 1984; Day and Fleck, 2015). The

morphological simplicity of these organisms, coupled with their cell wall characteristics help to maintain viability during the process. This was reflected by the high viability levels observed after the freezing, thawing and recovery cycle.

However it was observed that some strains of *Selenastrum bibraianum* had limited levels of recovery (Table 2.2) from the process. This demonstrated that even organisms very closely related may have specific requirements to ensure the complete success of the protocol (Brand *et al.*, 2013). This was also the case observed for some strains of *Kirchneriella,* specifically *K. aperta* and *K. pseudoaperta*, where somewhat limited success was observed on application of the standard two-step cryopreservation protocol using DMSO as CPA. *Kirchneriella pseudoaperta* (CCMA-UFSCar 346) when further tested demonstrated that the ability to form new cultures was maintained; however, the *lag* phase of the growth curve was a few days longer (see Chapter 5). Furthermore, a change in the protocol, i.e. employing MeOH as CPA during the two-step cryopreservation protocol resulted in slightly better recovery of viability (Fig 2.4), once again demonstrating the specific requirements which must be met for each culture to be cryopreserved. However, for most strains tested, no significant ($p>0.05$) differences were found in the protection provided by the CPAs tested (Fig 2.4), which can make them interchangeable for these organisms, giving priority to other aspects to be considered for the long term maintenance of frozen microalgae (Chapter 3).

2.5.3 Cryopreservation of desmids (Conjugatophyceae) and diatoms (Bacillariophyceae)

The organisms belonging to the Classes Conjugatophyceae (desmids) and Bacillariophyceae (diatoms) were some of the most recalcitrant groups for the protocols tested, with over 75% of the strains showing no post-thaw viability of samples. These groups have already been described as problematic for the maintenance in liquid nitrogen, with very few positive results for post-thaw viability (Day, 2007). Algae with larger cell sizes and most filamentous algae still cannot be cryopreserved yet, and the higher rates of success were found for smaller, morphologically non-complex algae (Day and Brand, 2005).

Successful cryopreservation protocols are known to reduce osmotic stress, cold shock and potential damage by ice formation, highlighting the importance of the cooling regime (Day *et al.*, 2000) and must be usually empirically determined for the more sensitive strains (Taylor and Fletcher, 1998). In a study with two strains of *Micrasterias,* Morris *et al*. (1986) reported that the cell walls did not contract to accompany the shrinkage of the protoplast, which led to the plasmolysis. This was not observed in similar studies with *Cylindrocystis brebissonii*, emphasizing the importance of cell volume and surface area, as well as possibly the cell wall composition as well as any interactions between the wall and cell membrane, and their influence on the cryopreservation process (Morris *et al.*, 1986).

2.5.4. Cryopreservation of *Chlamydomonas chlorastera*: a protocol study

The initial negative results for cryopreservation of *Chlamydomonas chlorastera* (CCMA-UFSCar 009) using DMSO as CPA in a two-step protocol indicated that further research was necessary for the successful long term maintenance of this strain. Thus, tests were performed using MeOH 5% and Glycerol 5%, as well as different cooling rates in order to obtain the highest viability results for these organisms. Although the cooling rates tested were not relevant for this algae, with the exception of the direct plunging of samples in liquid nitrogen, which was lethal independently of CPA, differences were found between CPAs, with MeOH being the only effective compound for the cyoprotection of this organism. Similar results were previously reported for tests with *Chlamydomonas reinhardtii* (Crutchfield *et al.*, 1999; Scarbrough and Wirschell, 2016; Yang and Li, 2016).

Neither of the CPAs tested had direct inhibitory effects on the growth of *C. chlorastera*, which leads to the indication of lethal damaging occurring at some point during the freezing/thawing of samples. Indeed, Yang and Li (2016) described, in tests with *C. reinhardtii*, that methanol was effective in attenuating the degradation of membrane lipids during the process, results that were not replicated with DMSO. As the complexity of the organisms to be maintained in liquid nitrogen storage increases, specific tests of protocol are necessary to reach the most adequate protocol, to avoid cryo-injury and other damaging effects of this stressful process for cultures.

2.5.5. Consequences of a cryopreserved biobank: perspectives for CCMA-UFSCar

The necessity for the establishment of a cryopreserved biobank is linked to several practical considerations including: the reduction of costs associated with routine transfers and the space necessary for the maintenance of cultures, the reduced risks of contamination and the maintenance of genetic integrity (Brand *et al.*, 2013). Thus, it is recommended that culture collections/Biological Resource Centers should maintain most of their holdings in cryopreserved stocks (OECD, 2007), to avoid the occurrence of genetic mutations, which will directly alter the characteristics of the cultures (Grout, 1995; Brand *et al.*, 2013). This is specifically true for strains of high commercial or scientific value, as only cryopreservation has the potential to guarantee their long term stability, including their potential to produce biotechnologically interesting products (Day, 2004; Hipkin *et al.*, 2014). CCMA-UFSCar microalgae are distributed as substrate for research throughout Brazil, and much data can be found on the importance and applicability of the maintained strains. Thus, the guaranteed stability offered by cryopreservation can provide insurance for the continuity of academic activities and possible applications of microalgae-derived compounds. Furthermore, as the collection is part of a laboratory in a Federal Institution, and not individually institutionalized, the maintenance personnel are mostly undergraduate and post-graduate students, who have to work in tandem with the development of their own research projects. Thus, the reduction of the cost and effort necessary for the constant sub-culturing is essential for the possibility of expansion of the culture holdings which was a result of the FAPESP Project efforts on the study of Brazilian biodiversity. Also, for the maintenance of cryopreserved stocks in liquid nitrogen dewars, the space required is reduced, another positive point in a university laboratory.

The positive results obtained for the initial pilot study on establishment of a cryopreserved biobank for CCMA-UFSCar illustrated the feasibility of introducing these practices for microalgae culture collections in Brazil, successfully conserving the largest part of the collection stocks, which as currently small unicellular organisms. However, it must be noted that some strains of microalgae are truly cryopreservation-recalcitrant due to the nature of their susceptibility to injury (Day, 2004). Also, some organisms require the dedicated studies in order to obtain the highest viability rates, due to specificities in their

biochemical composition and physical cell characteristics. Therefore the success observed using the protocols outlined in this study, whilst important cannot be applied to the full range of algal taxa held in the collection and further developmental work is needed to maximize the applicability of this approach to algal curation at CCMA-UFSCar.

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3 Cryopreservation of non-axenic strains at UFSCar Culture Collection of Freshwater Microalgae (CCMA-UFSCar)

3.1 Abstract

Culture collections of microalgae usually maintain their holdings the format of unialgal cultures, which still contain the algae-attached/associated bacterial heterotrophic community. Although some applications require axenicity of cultures, i.e. cultures free of all contaminants, to insure the reliability and reproducibility of tests, interest on the use of non-axenic cultures has been growing, particularly for application on the remediation of wastewater and production of high-value products. In this study, the standard cryopreservation process tested for non-axenic strains had, in general, high levels of viability, which were comparable to the results for axenic strains previously reported. However, adequate care must be taken to insure the predominance of the microalgae culture over the heterotrophic bacterial community, as the proliferation of the latter might inhibit the growth of the target strain, as observed for tests of cryopreservation at different growth phases of the culture. As part of the services provided by culture collections, purification of target organisms, employing antibiotics, might be required for furthering application in research or for biotechnological exploitation. However, combination of this process with cryopreservation techniques, without allowing the required recovery time could reduce or nullify viability for some strains of microalgae after the completed freezing/thawing cycle.

3.2 Introduction

The culture generated from the process of isolation of organisms from environmental samples until a single strain of microalgae is present is defined as unialgal culture (Guillard, 2005). This is the format in which most strains are usually maintained in culture collections. However, the mechanical process of isolation is not usually enough to eliminate the attached heterotrophic community (Brand *et al.*, 2013). Contaminated cultures, or more appropriately defined as non-axenic cultures, might be undesirable for genetic analysis, specific

biotechnological exploitation and derivation of some microalgae products (Day *et al.*, 2012; Brand *et al.*, 2013). Some of these can require cultures free of contaminant living organisms, which are called axenic cultures (Guillard, 2005).

Culture collections are always in expansion mode, which requires opening space for new strains to be accessed, usually coming either by the isolation of strains from collected field samples or by the deposition from external providers (Brand *et al.*, 2013). Thus, most of the collection holdings are maintained as unialgal cultures until required otherwise (Guillard, 2005; Lorenz *et al.*, 2005), due to the elevated risks of maintaining axenic cultures and the difficulty of the process for determined strains. Furthermore, the requests of non-axenic cultures has been expanded, as their potential was observed for algal-bacterial association studies (Ueda *et al.*, 2009), and some algae-bacteria consortia were also found useful for application in food industry and wastewater treatment (Watanabe *et al.*, 2005). Howewer, the conservation of non-axenic strains using cryopreservation can be problematic, as the recovery of samples will be affected by the "contaminant" organisms (Amaral *et al.*, 2013). The choice of cryoprotectant might be a key-factor for the successful recovery of samples, due to their cryoprotective activity (Hubalek, 2003) and also, if sugar based, their function as substrate for the heterotrophic bacterial community (Amaral *et al.*, 2013). Also, there are risks during the process, as the contamination from external organisms might affect the ongoing balance of the cultures, which will have effect on culture growth and physiology of cultures, including production of metabolites (Day *et al.*, 2012). Currently, the Culture Collection of Freshwater Microalgae (Coleção de Culturas de Microalgas de Água-doce - CCMA-UFSCar) at Universidade Federal de São Carlos holds 688 strains of microalgae. From these, only around 20% (133 strains) are maintained as axenic cultures, which raise the necessity for the establishment of protocols for freezing and recovery of these strains without loss of viability and growth characteristics.

Although the possibilities for non-axenic strains have grown, the purification of strains might still be requested as one service provided by culture collections, or even to guarantee the correct identification of the samples maintained. Obtaining an axenic microalgae culture is usually a laborious and time-consuming process, which will not always reach positive results, since some organisms are reliant on their attached/associated

bacterial community for survival (Droop and Elson, 1966). Also, the removal of contaminant bacteria from microalgal cultures requires mechanical and/or chemical (antibiotics) processes, although the latter is usually contested as it may cause cellular damage during the process (Droop, 1967).

It is widely recognized that the cryopreservation process can be stressful for microalgae cultures, with many opportunities for damage during freezing and thawing of strains (Day and Fleck, 2015). For this study, the effects of contaminant bacterial community and cryoprotectant choice on the post-thaw viability and recovery of cultures of microalgae were analyzed, together with how possible changes on the community during the growth phases may affect the survival of samples. Furthermore, as the purification of non-axenic strains is often required for biotechnological or physiological studies, the direct effects of the combination of the cryopreservation process with the treatment of strains with antibiotics on the survival of strains was assessed for a few species of microalgae.

3.3 Material and Methods

3.3.1 Post-thaw viability: non-axenic cultures

3.3.1.1 Organisms

For this test, 18 strains of microalgae were chosen (Table 3.1). These organisms are currently maintained in unialgal cultures for the main collection, and were recently purified to axenic cultures to facilitate further laboratory research. The responses of these axenic cultures to cryopreservation with two different CPAs were reported in Chapter 2. Non-axenic cultures of these strains were grown in WC medium (Guilard and Lorenzen, 1972) under 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark : light cycle and at a temperature of 23 ± 1^{o}C.

Table 3.1 - Organisms selected for analysis of post-thaw viability levels and comparisons between cultures axenic and non-axenic cultures.

Reference number		Species
CCMA-UFSCar	**046**	*Desmodesmus spinosus*
CCMA-UFSCar	**047**	*Selenastrum bibraianum*
CCMA-UFSCar	**048**	*Raphidocelis subcapitata*
CCMA-UFSCar	**060**	*Coelastrum sphaericum*
CCMA-UFSCar	**062**	*Desmodesmus spinosus*
CCMA-UFSCar	**140**	*Monoraphidium griffithii*
CCMA-UFSCar	**325**	*Monoraphidium pseudobraunii*
CCMA-UFSCar	**333**	*Monoraphidium kormakovae*
CCMA-UFSCar	**345**	*Kirchneriella obesa*
CCMA-UFSCar	**346**	*Kirchneriella pseudoaperta*
CCMA-UFSCar	**348**	*Kirchneriella irregularis*
CCMA-UFSCar	**387**	*Chlorolobion guanense*
CCMA-UFSCar	**390**	*Chlorolobion lunatum*
CCMA-UFSCar	**399**	*Botryococcus braunii*
CCMA-UFSCar	**443**	*Kirchneriella lunaris*
CCMA-UFSCar	**487**	*Hariotina reticulata*
CCMA-UFSCar	**607**	*Pediastrum duplex*
CCMA-UFSCar	**609**	*Desmodesmus comunis*

3.3.1.2 Experimental design

Cryopreservation in triplicates for each culture concentration were performed using a two step protocol (Day and Brand, 2005) using 5% dimethyl sulfoxide (DMSO), or methanol (MeOH), as the cryoprotectant additive (CPA). Samples were thawed after 3-4h in liquid nitrogen, immediately diluted in 10 mL fresh sterile WC medium and incubated under 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark : light cycle and at a temperature of 23 ± 1^{o}C. Viability of samples was assessed using vital staining (erythrosine-b) after 24h and viability levels were calculated as described in Chapter 2. In addition, visual assessment on the recovery of samples was performed after 2 weeks of growth in fresh WC medium.

3.3.2 Effect of CPA and growth phase on the success of recovery of cryopreserved non-axenic strains

3.3.2.1 Organisms

The effect of CPA and culture age (growth phases) on the cryopreservation of non-axenic cultures was tested for four strains maintained at the Culture Collection of Freshwater Microalgae at Universidade Federal de São Carlos (CCMA-UFSCar). The species/strains tested were *Haematococcus pluvialis* (CCMA-UFSCar 344), *Sphaerocystis schroeteri* (CCMA-UFSCar 396), *Eutetramoris fottii* (CCMA-UFSCar 212) and *Pseudokirchneriella elongata* (CCMA-UFSCar 498), all were maintained in metabolically active cultures, growing in WC medium (Guilard and Lorenzen, 1972) under 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark : light cycle and at a temperature of 23 ± 1^{o}C. For cryopreservation tests, cultures were grown in 125 mL Erlenmeyer flasks containing 75 mL of WC medium.

3.3.1.2 Effect of the culture age (growth phase)

This test was performed to analyze the differences in the recovery of samples cryopreserved during the exponential (2 weeks) and stationary (5 weeks) phases of growth. For this, after the determined periods of growth, triplicate samples for each culture were cryopreserved using a two step protocol (Day and Brand, 2005), using 5% dimethyl sulfoxide (DMSO), or methanol (MeOH), as the cryoprotectant additive (CPA). Samples were thawed after 3-4h in liquid nitrogen, and diluted in 10 mL fresh sterile WC medium and incubated for growth in similar to banking conditions. The viability of samples immediately assessed using Chl-a fluorescence readings, and recovery was analyzed using visual analysis of growth (photographs) and microalgae growth rates, which was analyzed in Chl-a fluorescence measurements using a Trilogy Laboratory Fluoremeter (Turner Designs, USA) equipped with a chlorophyll *in vivo* Module (7200-043). Control cultures (not frozen) were tested under similar conditions (initial cell concentration) to the frozen samples, as well as tests for the effects of treatment o samples with CPAs prior to cryopreservation. In addition, the viability

of samples immediately after thawing was also assessed using vital staining (erythrosine-b). The percentages of viability were calculated as described in Chapter 2.

3.3.3. Effect of antibiotics on the cryopreservation process and recovery of non-axenic cultures

3.3.3.1. Organisms

In this study, four non-axenic strains were selected from CCMA-UFSCar. The axenic cultures derived from the unialgal cultures were also available from the collection holdings. The selected strains were: two strains of *Desmodesmus spinosus* (CCMA-UFSCAR 046 and CCMA-UFSCar 062), *Selenastrum bibraianum* (CCMA-UFSCar 168) and *Nephroclamys subsolitaria* (CCMA-UFSCar 132). Two separate cultures of each strain (one axenic and one non axenic) were grown for 2 weeks in preparation for the tests in 125 mL Erlenmeyer flasks containing 70 mL of WC medium, and incubated under 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark : light cycle and at a temperature of 23 ± 1^{o}C.

3.3.3.2. Experimental design

The utilization of antibiotics during the preparation of samples for cryopreservation and the recovery of frozen samples, and its effect on the recovery of samples, was assessed in a test which combined different treatments:

T1 – Axenic Control

T2 – Axenic + Frozen Sample

T3 – Non-Axenic Control

T4 – Non-Axenic + Frozen Sample

T5 – Antibiotics Control

T6 – Antibiotics + Frozen Sample

T7 – Non-Axenic + Frozen + Antibiotics Recovery

Where the treatments were:

Axenic: strain previously purified and maintained in axenic conditions at the biobank;

Non-Axenic: Same strain, maintained with the isolated bacterial community attached;

Frozen: Standard two step freezing protocol as described (Day and Brand, 2005), using DMSO as CPA. After thawing samples were diluted in culture tubes containing 10 mL fresh WC medium;

Antibiotics: Non axenic strains were concentrated by centrifugation and treated, previously to the cryopreservation procedures, with a penicillin+streptomycin solution in WC medium, with final concentration of 50 U mL^{-1}, during 4h. After exposure to the antibiotics, samples were diluted with WC medium in order to obtain cell concentrations similar to the original culture;

Antibiotics Recovery – After the freezing and thawing cycle, 1mL samples were diluted in fresh sterile WC medium containing 50 U mL^{-1} of penicillin+streptomycin.

All treatments were assembled in triplicate cultures for each strain tested. After treatment samples were maintained for 36h in the dark and subsequently incubated for growth under the normal environmental conditions, as described for the strains in the collection catalogue. Algal growth was assessed using Chl-a fluorescence measurements in a Trilogy Laboratory Fluoremeter (Turner Designs, USA) equipped with a chlorophyll *in vivo* Module (7200-043).

3.3.4 Statistical Analysis

When applicable, comparisons between each treatment and control were performed using a non-parametric ANOVA (Kruskal-Wallis). When differences were significant ($p<0.05$), t-tests were used to compare each sample with control, to determine the treatments where significant differences occurred.

3.4 Results

Post-thaw viability levels observed for non-axenic cultures were, in general, high and similar to the responses reported for axenic cultures of the same strain frozen in equal conditions (Chapter 2). Comparisons for the viability of non-axenic strains frozen with MeOH and DMSO as CPA are detailed in Fig. 3.1.

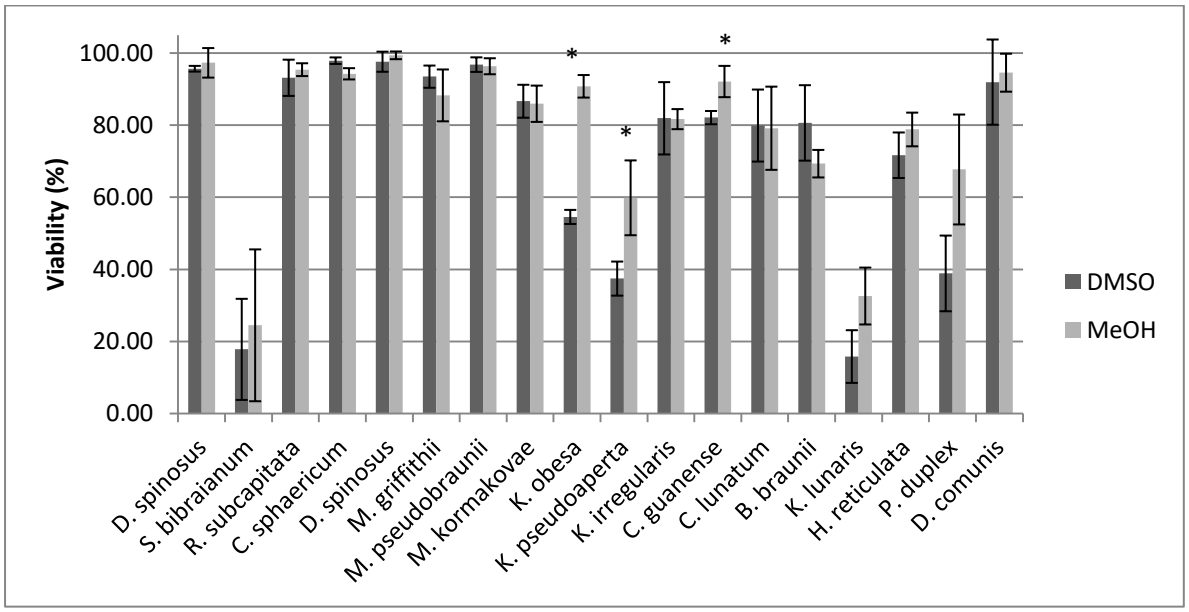

Figure 3.1 - Post-thaw viability rates obtained for non-axenic strains cryopreserved using DMSO or MeOH as CPA. *= statistically significant differences (p<0.05)

It was noted that for a few strains, mostly of *Kirchneriella* spp*,* significantly higher viability was observed with samples treated with MeOH for cryopreservation. The ability to form cultures from these samples was visually assessed and the results can be observed in Fig. 3.2. Most results obtained on employing vital staining were confirmed by the growth of the cultures; however, cultures of *M. pseudobraunii* treated with MeOH showed severe inhibition of growth, when compared to control cultures of the same organisms. Both *K. obesa and K. pseudoaperta* had reduced viability after freezing using DMSO as CPA, as already noted using staining (Fig 3.1 and Fig. 3.2). Although viability of *H. reticulata, P. duplex* and *D. comunis* was high, the development of these cultures was slower than the other strains tested, and results of limited growth were observed after 2 weeks (Fig. 3.2).

However, all samples from these strains were still able to form viable cultures after 4 - 6 weeks of growth in liquid media, denoting a high regeneration ability of these cultures (data not shown), not hindered by the attached bacterial community.

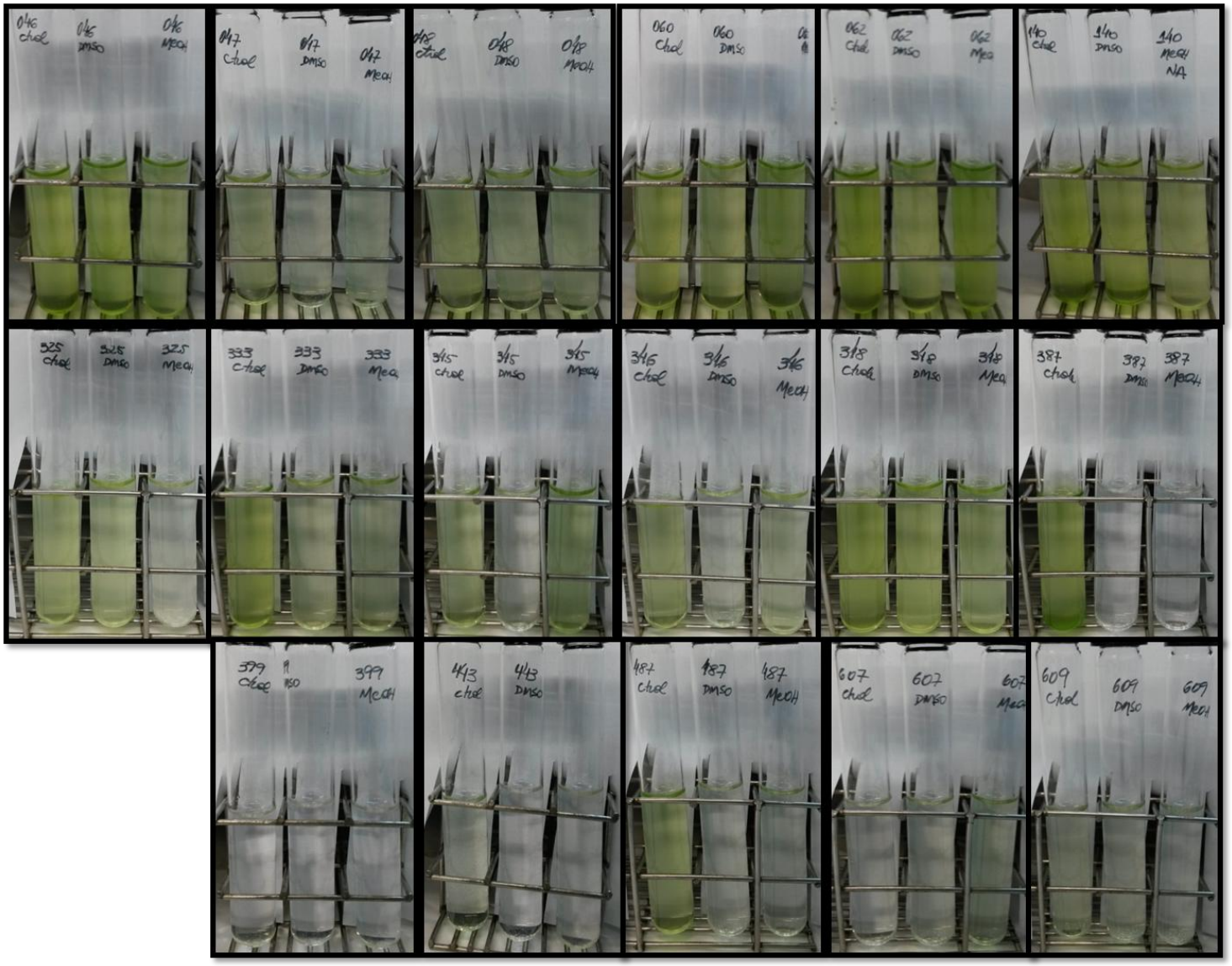

Figure 3.2 - Visual analysis of growth of non-axenic cultures of freshwater microalgae 2 weeks after cryopreservation. From left to right and top to bottom: *Desmodesmus spinosus* (046); *Selenastrum bibraianum* (047); *Raphidocelis subcapitata* (048); *Coelastrum sphaericum* (060); *Desmodesmus spinosus* (062); *Monoraphidium griffithii* (140); *Monoraphidium pseudobraunii* (325); *Monoraphidium komarkovae* (333); *Kirchneriella obese* (345); *Kirchneriella pseudoaperta* (346); *Kirchneriella irregularis* (348); *Chlorolobium guanense* (387); *Botryococcus braunii* (399); *Kirchneriella lunaris* (443); *Hariotina reticulata* (487); *Pediastrum duplex* (607); *Desmodesmus communis* (609).

The development of a protocol for the cryopreservation of non-axenic strains must consider the bacterial effect on the recovery of liquid cultures, as the bacterial community can have better recovery strategies, which could potentially lead to inhibition of the growth of the microalgae cultures (Amaral *et al*., 2013). Although the CPAs had little effect on the development of *Haematococcus pluvialis,* the cryopreservation protocol led to damage to the cell, with loss of viability in the frozen samples, which caused delayed development on the cultures and lower growth rates (Fig. 3.3). Both growth phases tested showed similar responses to treatments, with no significant differences being observed between samples grown for 2 or 4 weeks before cryopreservation ($p>0.05$).

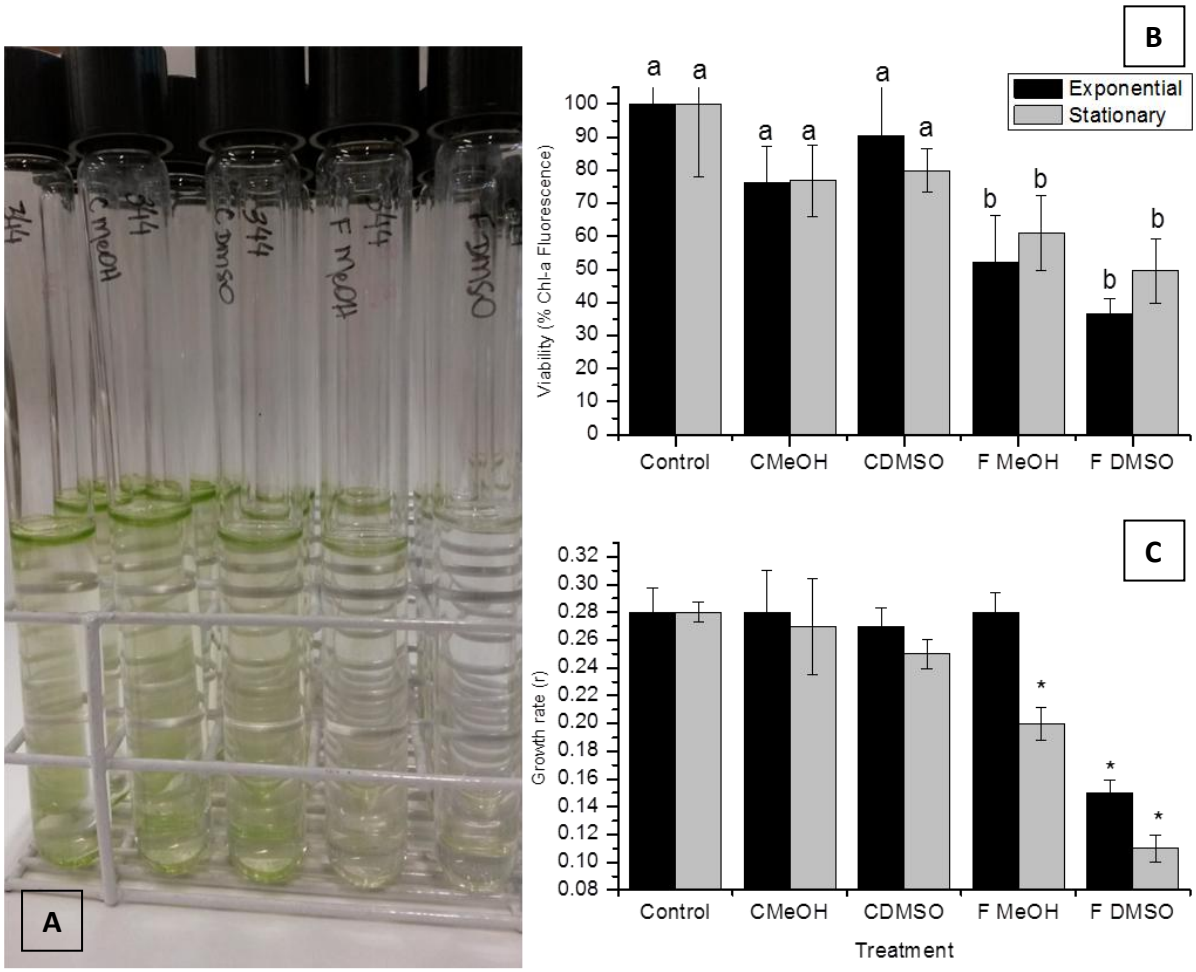

Figure 3.3 - Post-treatment cultures regenerated from stationary phase inoculum (A), Viability (B) and growth rates (C) of post-thaw cultures of *Haematococcus pluvialis* (CCMA-UFSCar 344). The C treatments are control cultures after exposure to CPA and F are cultures frozen after treatment with CPA.

For cultures of *Sphaerocystis schroeteri*, although no large effects were observed on the viability of samples after cryopreservation (Fig. 3.4-B), the growth of microalgal samples treated with MeOH was severely inhibited for those cultures cryopreserved during the stationary phase of growth. These cultures also had visible growth of the bacterial community (development of opacity in the medium), which could have affected the development of the microalgae (Fig. 3.4).

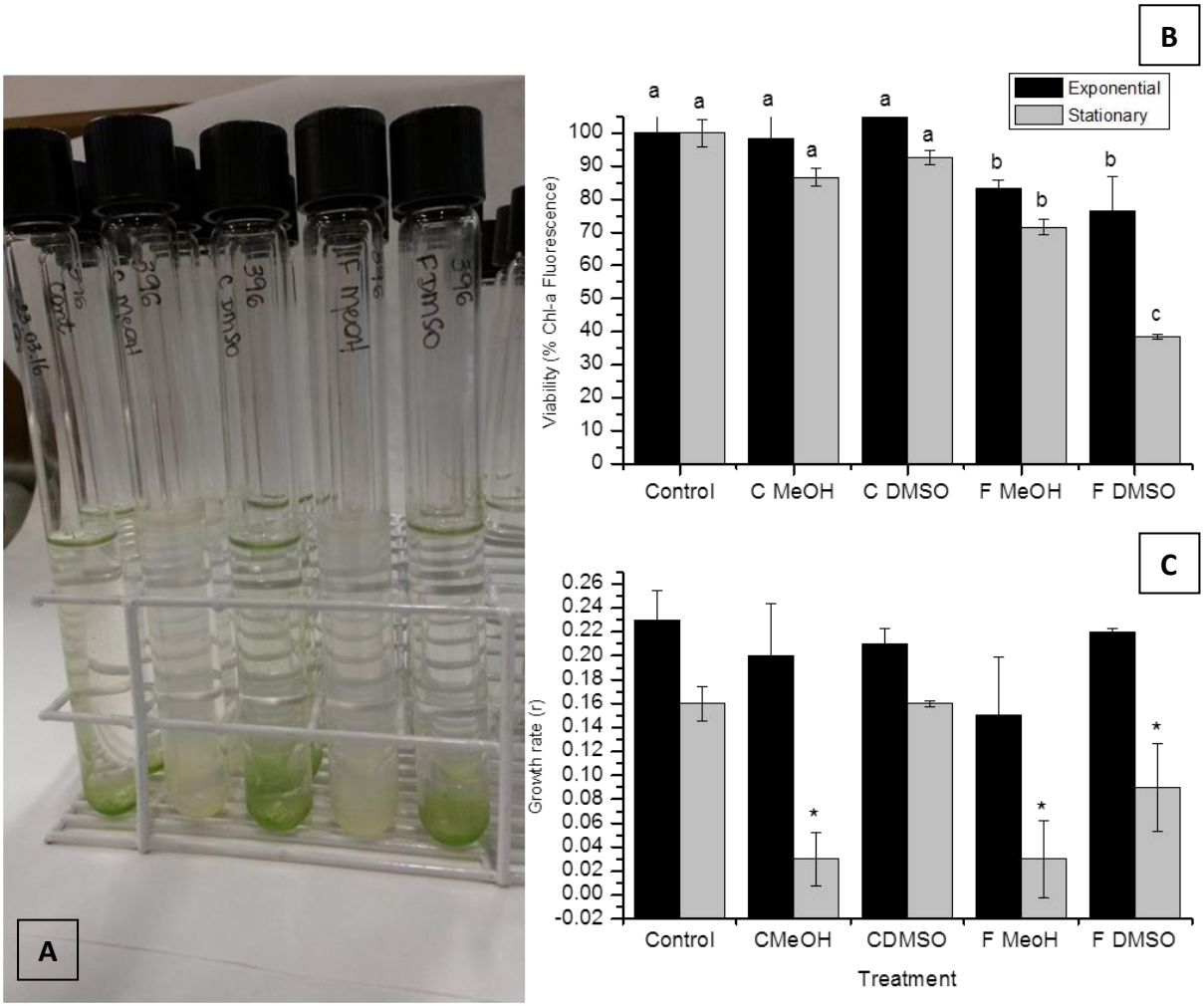

Figure 3.4 - Post-treatment cultures from stationary phase inoculum (A), Viability (B) and growth rates (C) of post-thaw cultures of *Sphaerocystis schroeteri* (CCMA-UFSCar 396). The C treatments are control cultures after exposure to CPA and F are cultures frozen after treatment with CPA.

Eutetramoris fottii had slower growth of the older samples, in general and statistically significant differences were observed on the growth of cultures treated with DMSO, which was slightly higher than control and a small reduction on growth of cryopreserved samples treated with MeOH (Fig. 3.5). Also, for this strain of microalgae, damaging effects of the cryopreservation process, and reduced viability were observed for both culture ages tested. However, with the exception of the older cultures treated with MeOH, all samples were able to rapidly overcome these effects, with subsequent fast development of robust cultures after thawing.

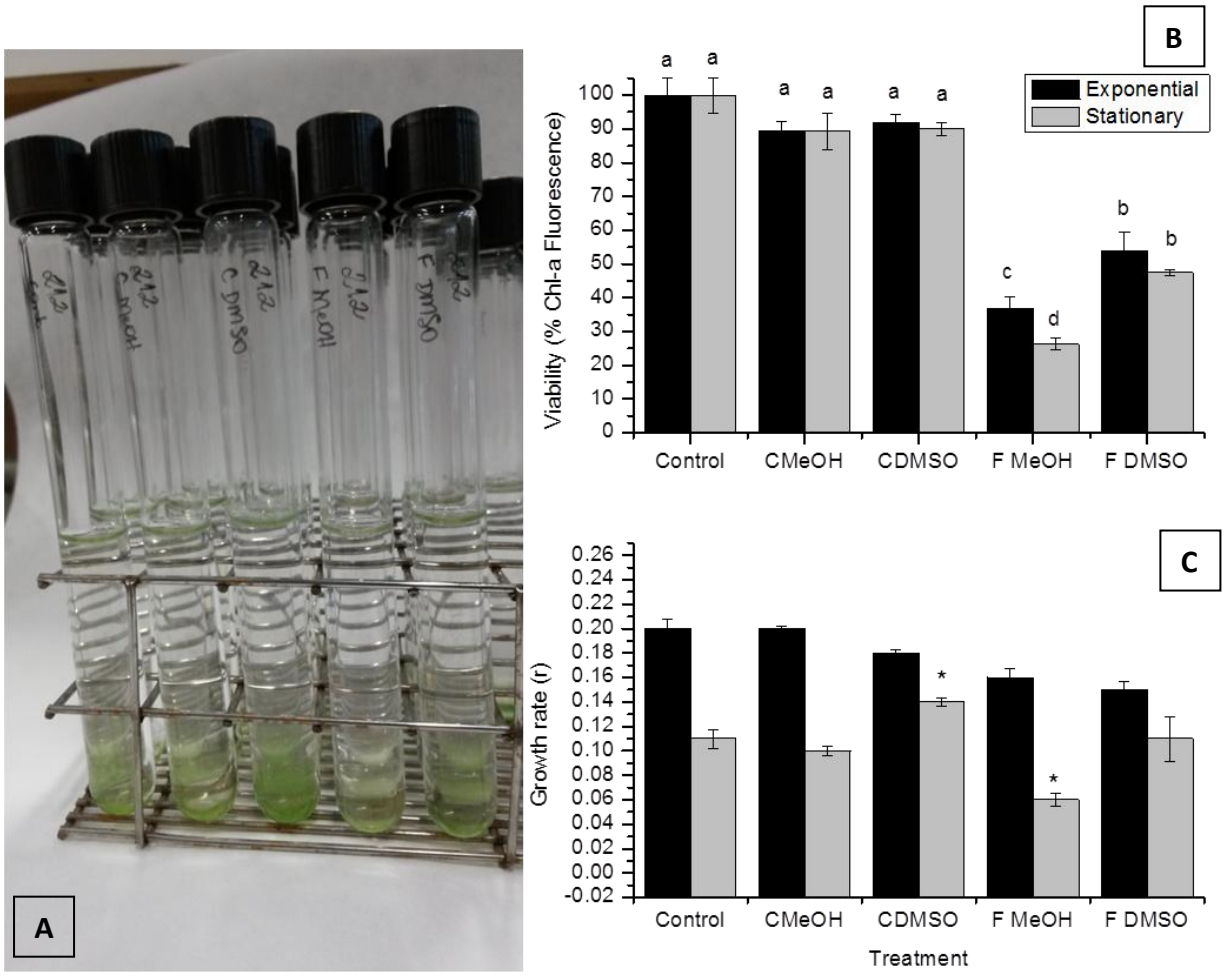

Figure 3.5 - P -treatment cultures from stationary phase inoculum (A), Viability (B) and growth rates (C) of post-thaw cultures of *Eutetramoris fottii* (CCMA-UFSCar 212). The C treatments are control cultures after exposure to CPA and F are cultures frozen after treatment with CPA.

For the cultures of *Pseudokirchneriella elongata* (Fig. 3.6), although no pronounced differences in viability were observed for post-thaw cultures, the growth rates for samples tested after 4 weeks of growth (stationary phase) were lower on post-thaw recovery, when compared to the results observed for samples in exponential growth. Also, the visual inspection of the samples indicated the development of opacity on the samples treated with MeOH (Fig. 3.6A); however, this had no significant ($p<0.05$) effects on the development of the microalgae.

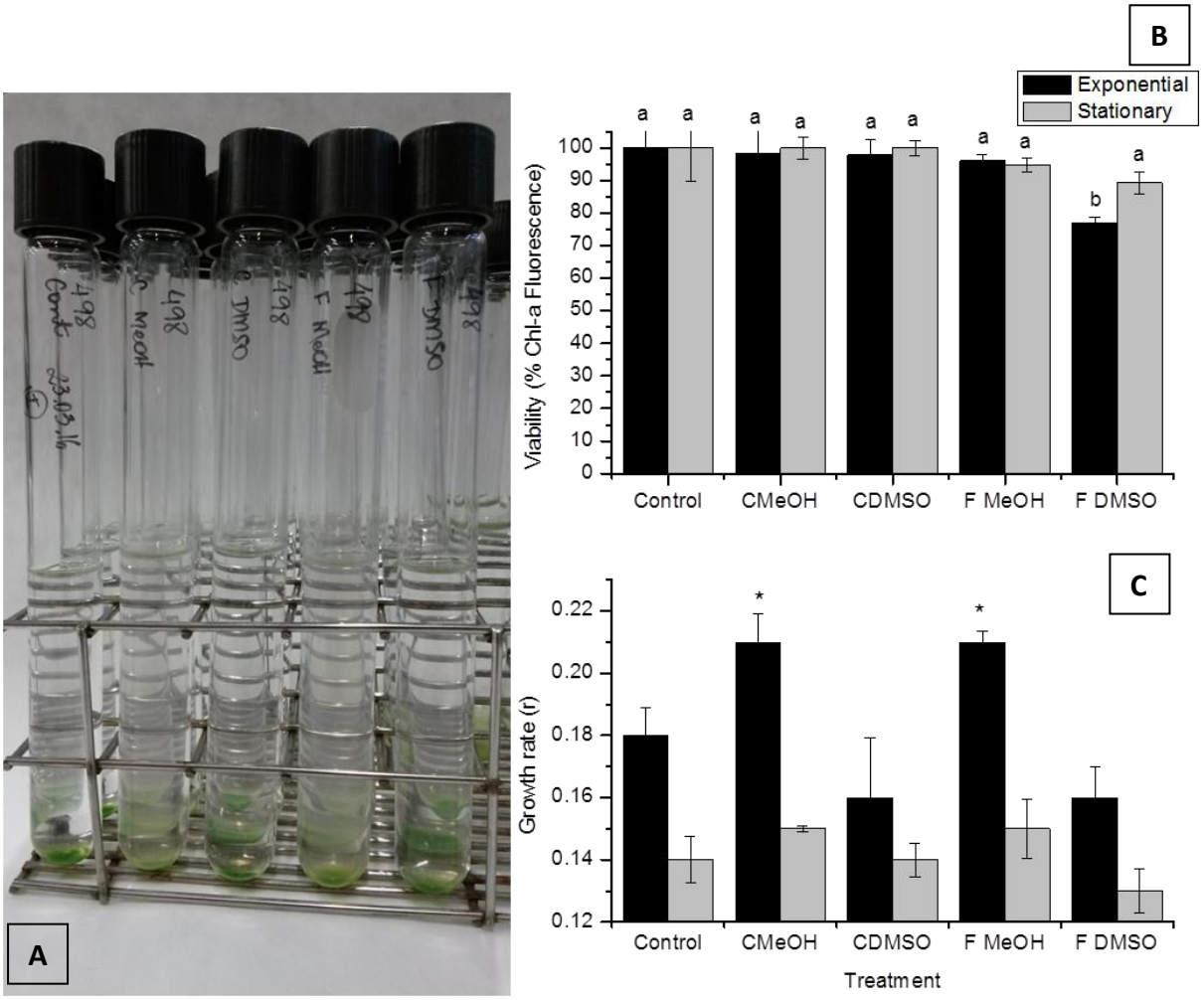

Figure 3.6 - Post-treatment cultures from stationary phase inoculum (A), Viability (B) and growth rates (C) of post-thaw cultures of *Pseudokirchneriella elongata* (CCMA-UFSCar 498). The C treatments are control cultures after exposure to CPA and F are cultures frozen after treatment with CPA.

On testing the effects of antibiotic treatment on *Desmodesmus spinosus*, no effect on the growth of samples, and also, no differences were found on the survival rates of these samples to the freezing/thawing process. However, trials with the recovery of samples cryopreserved in treated medium were not successful, probably due to the higher frailty of samples after the process (Fig 3.7 A,B).

For the strain of *Nephroclamys subsolitaria* tested, although this culture is currently maintained in axenic conditions at CCMA-UFSCar, and both axenic and non-axenic samples had similar responses to the freezing protocol, the exposure of these samples to treatment with antibiotics led to the loss of viability, which were not able to form new cultures after treatments. Similar results were observed for *Selenastrum bibraianum*, which although low levels of survival were observed after antibiotic treatment, no survival was observed after the combined effects of this treatment with the damaging effects of the cryopreservation protocol.

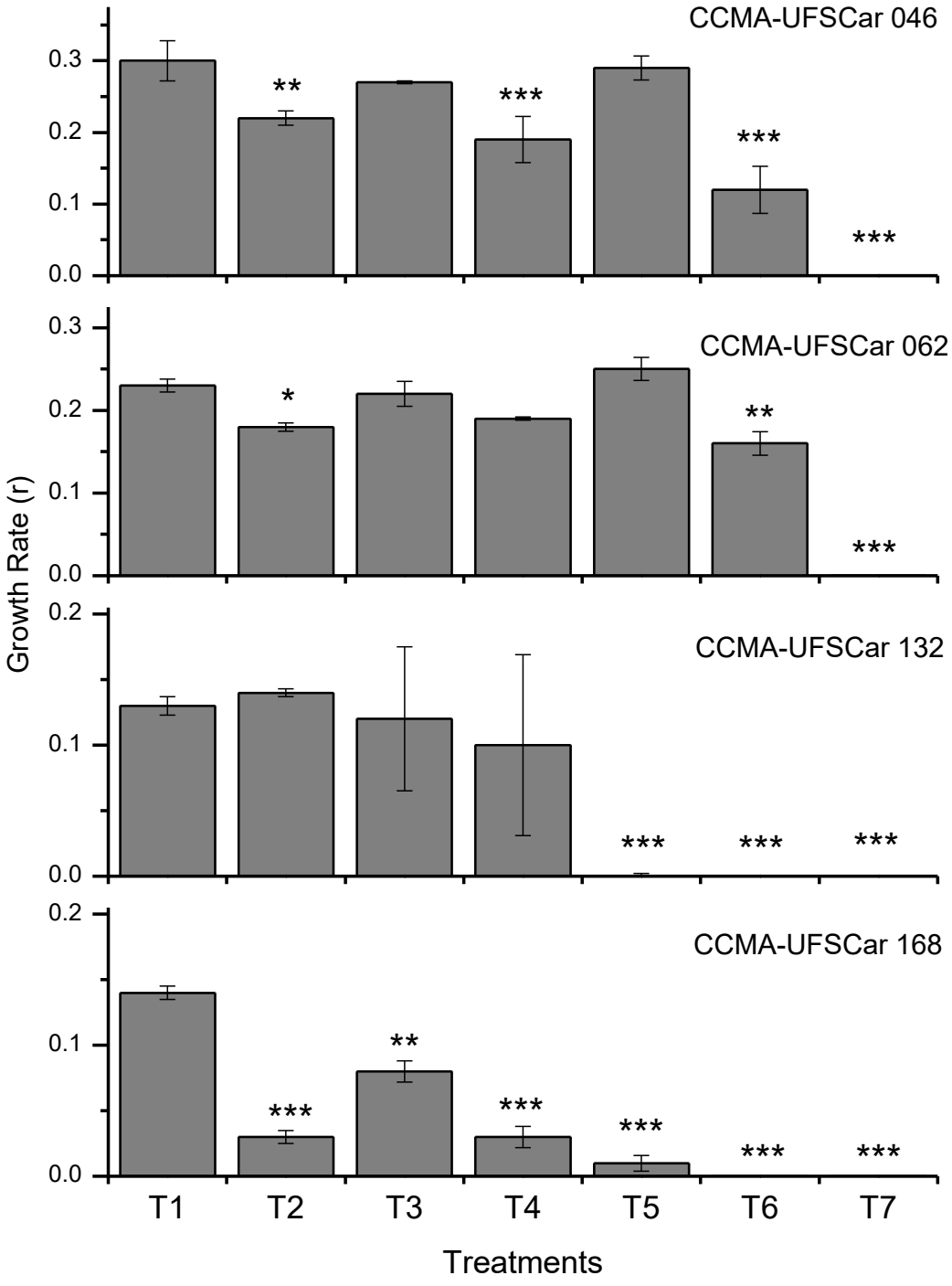

Figure 3.7 - Viability of cultures, assessed indirectly using growth rates (r) for organisms of (a) *Desmodesmus spinosus* (CCMA-UFSCar 046), (b) *Desmodesmus spinosus* (CCMA-UFSCar 062), (c) *Nephroclamys subsolitaria* (CCMA-UFSCar 132) and (d) *Selenastrum bibraianum* (CCMA-UFSCar 168) after a variety of treatments: T1 - Axenic Control; T2 – Axenic Frozen; T3 – Non-Axenic Control; T4 – Non-Axenic Frozen; T5 – Antibiotics Control; T6 – Antibiotics Frozen; T7 – Non-Axenic Frozen and Recovered in medium with Antibiotics.

*** = statistically significant difference: p<0.05; ** = p<0.01; *** = P<0.001**

3.5 Discussion

As detailed in the introduction to this chapter, in most cases acquisition and maintenance of microalgae strains in collections involves unialgal cultures, which inevitably means that most still contain their original associated bacterial community (Brand *et al.*, 2013). For the cryopreservation and long term maintenance of these cultures, although axenicity would be desirable for the facilitation of effective phenotypic and genotypic stability tests, this characteristic is not a crucial aspect of the process *per se* (Amaral *et al.*, 2013). Indeed, in this study, the post-thaw viability levels obtained for the non-axenic strains tested were generally high (Fig. 3.1) and directly comparable to their axenic counterparts reported previously (Chapter 2). For *Kirchneriella obesa*, *K. pseudoaperta* and *C. guanense,* viability was higher for samples cryopreserved using MeOH as CPA ($p<0.05$), rather than DMSO. Similar results were observed, although with slightly less pronounced differences, for the axenic strains (Chapter 2). It is important to note that most of these cultures tested are unicellular, small Chlorophyceaen algae, which generally have expected high successful rates of survival after cryopreservation considering their size and morphological simplicity (Day and Brand, 2005).

The process of cryopreservation of non-axenic strains, beyond the viability of samples assessed immediately after the freezing/thawing cycle, must also consider the ability of samples to recover and overcome competition with co-culture organisms. The propagation of the heterotrophic associated community may lead to inhibition of microalgae growth (Day and Harding, 2008), and, therefore, the choice of CPA was demonstrated to have direct effect on the recovery of robust cultures of microalgae of non-axenic strains. The utilization of MeOH as cryoprotectant during this process might be problematic, as although it results in higher viability as demonstrated for some strains of *Kirchneriella* (Fig. 3.1), it also favors the proliferation of bacteria, verified by the opacity developed in the medium. The conditions resulting in the appearance of higher bacterial concentration after cryopreservation might be related to the release of cell contents by disruption due to the stress during the process, which may be used as substrate for other organisms (Harding *et al.*, 2010). Furthermore, MeOH is a possible source of carbon for microorganisms, which

could help the proliferation of the heterotrophic community to the detriment of the microalgae (Amaral *et al.*, 2013).

The effects of the choice of CPA were also observed for the tests with *H. pluvialis*, *S. schroeteri* and *Eutetramorus fottii* samples from two different phases of the growth, and these results were even clearer when analyzing the older cultures (stationary phase), with visible opacity observed due to proliferation of the associated bacterial flora. Whilst for some algae the presences of bacteria was not deleterious, proliferation of the bacterial community during post-treatment culture establishment can have a detrimental effect on microalgae if their recovery rates are slower, with inhibitory effects due to competition for nutrients and in extreme cases alteration of the pH of their culture environment (Day and Harding, 2008).

The majority of CCMA-UFSCar cultures are maintained as unialgal cultures, as obtained after isolation from environmental samples, thus, most still have an associated bacterial community. For the organisms tested in this study, most cryopreservation constraints associated with non-axenic cultures seem to be easily overcome. Maintaining samples under these conditions is usually more desirable for culture collections, as it reduces the need for additional purity checks that require both manpower and consumables. Furthermore, recently the value of algal/bacterial co-cultures have been demonstrated (Ueda *et al.*, 2009) and their exploitation for wastewater treatment and production of commercial compounds has been studied, with increased productivity of biomass and fatty acids by some non-axenic strains being reported (Halfhide *et al.*, 2014). The maintenance of large scale cultures in axenic conditions is difficult and expensive, and the costs of maintaining axenic cultures for some large scale biotechnological applications might overcome the products value (Wilkie *et al.*, 2011), reducing the incentives for obtaining pure cultures. The maintenance of non-axenic strains in culture collections might also offer foundation for important physiological studies, as already reported that the production of high value metabolites can be different in axenic and non-axenic cultures (Wang *et al.*, 2016), for example, the profiles of saturated and unsaturated fatty acids in wastewater

isolated strains, for which the non-axenic cultures produced the most profitable blends (Stemmler *et al.*, 2016).

However, despite the obvious issues involved, there remains a strong rationale for maintaining axenic cultures including the need to comply with standards across the BRCs community, ensuring the correct identification of samples, the "purity" of the organism and offer of target organisms for furthering research in a wide range of subjects (OECD, 2007). The axenicity of the microalgae strains might also become a requirement to provide reliable and replicable results in specific situations (Olaizola, 2003; Day *et al.*, 2010). Thus, services of purification might be necessary and provided from the culture collection or to be applied by receiver before their direct application for research purposes (Amaral *et al.*, 2013).

The purification of microalgae is usually a time consuming and delicate process particularly when dealing with samples recently isolated. From experience it can take in excess of 6 months to ensure axenicity and some taxa are apparently incapable of surviving without their associated flora, even when vitamins and other growth factors are provided (Day, pers. Comm.). One of the usual approaches employed for the purification of cultures involves the use of antibiotics (Guillard, 2005) which will act against the proliferation of prokaryotes and fungi. The effect of antibiotics immediately before and after the cryopreservation protocol on four species of microalgae in this study demonstrated that the combined effects of these two stressful protocols were lethal for some microalgal cultures, or were at least severely debilitating (Fig. 3.7). Considering the four strains of microalgae tested in this study, three of them were able to survive the treatment with antibiotics, with the strains of *D. spinosus* being the more resistant, as no changes were found for the growth of these cultures after treatment. Cryopreservation of these strains maintained their ability of growing new cultures after the freezing cycle, although their vigor was slightly reduced after both treatments were combined (Fig. 3.7 – T6). The strain of *N. subsolitaria* tested was the most susceptible for the antibiotics treatment, which inhibited the growth of microalgae after exposure. For the strain of *S. bibraianum,* reduced growth of samples was still observed after treatment with antibiotics. However, the cryopreservation of this strain was also found to have a damaging effect on the growth of cultures untreated before the process. The

combination of the damaging effects of the antibiotics and cryopreservation protocol were lethal for samples of all the strains tested and no recovery after these treatments was observed. Incubation of cryopreserved samples recently recovered to growth in antibiotics inoculated medium also resulted in inhibition of growth on all strains tested.

The antibiotics tested in this study combined one cell wall inhibitor (penicillin) and one protein synthesis inhibitor (streptomycin), which is often used in avoiding contamination of cell cultures. Such cocktails have been often successful for eliminating contamination in microalgae cell cultures (Guillard, 2005). This combination has also been used for purification on *D. spinosus* strains previously at CCMA-UFSCar, with success in obtaining bacterial free cultures from these strains (Bagatini, IL – pers. comm.). However, the concentrations necessary for the effective action of these compounds might also affect the viability of microalgae cultures, causing damage to cell wall and consequently cell death (Cho *et al.*, 2013). This directly affects the susceptibility of an individual alga to cryopreservation induced damage, resulting in the process being lethal for most microalgal cultures. The separation of the process of cryopreservation and purification of cultures seems to be necessary to ensure successful recovery of samples, due to the intrinsic stress caused by both these procedures on the growth of microalgae samples. Therefore an appropriate length of recovery time for cultures cryopreserved must be provided before attempts of purification on these strains are considered.

Although the combination of the purification and the freezing processes resulted in reduced success for the strains tested, their cryopreservation and maintenance as stand-alone processes are highly viable and recommended for culture collections, since both axenic and non-axenic strains have their intrinsic value and applications. However, careful approaches must be considered to avoid the extreme proliferation of the bacterial community and the contamination with exogenous organisms, which could and will affect the population dynamics and metabolism of microalgae (Grossart and Simon, 2007; Wang *et al.*, 2016).

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4 Influence of temperature of storage on the longevity of cryopreserved samples of freshwater microalgae

4.1 Abstract

Cryopreservation, i.e. the storage of viable samples at ultra-low temperatures, is currently accepted as the most reliable system for maintaining microalgae cultures without loss of viability and cells characteristics for long periods of time. However, the temperature and stability of storage environment can have profound effects on the quality of samples and their ability to regrow cultures, as it will influence the ice formation and, consequently, the possibility of cryoinjuries. Here, the effect of three different temperatures on the storage of four microalgae strains frozen using two different cryoprotective additives (CPAs) were studied during the period of one year. The results showed severely reduced or no viability on some samples maintained at suboptimal temperatures. Also, the use of different CPAs was found to influence the response of samples to maintenance in temperatures over -130^{o}C, as we verified viability loss using methanol as cryoprotective for samples maintained at -80^{o}C. The maintenance of samples in liquid nitrogen (LN_2) insured the sustained high viability throughout the year. Furthermore, a case study of strains maintained for long-term storage in LN_2 for up to 40 years at the Culture Collection of Algae and Protozoa reinforced this method as a reliable technique for the long term maintenance of samples after successful freezing, independently of the freezing protocol applied. However, the management and quality control on cryopreserved biobanks must be rigorously implemented, as differences in temperature may lead to the loss of important samples in a very short time-frame.

4.2 Introduction

Cryopreservation is the storage of viable cells and organisms at ultra-low temperatures, usually in liquid nitrogen (LN_2 - temperature around -196^{o}C). This technique is currently considered the most effective option for the long term storage of microalgae cultures without loss of morphological and biochemical characteristics, as it allows the

organisms that do not have any natural resting stage to be maintained indefinitely in an "arrested state" (Day and Brand, 2005). Thus, it ensures their consistent availability for the ever growing demand for environmental, toxicological and biotechnological studies (Day and Harding, 2008).

The most basic principle of cryopreservation lies on the concept of *´freezing biological time´*, i.e. the vital functions of cells are slowed, to the point of cessation, by exposure to ultra-low temperatures (Benson, 2008). The success of a cryopreservation protocol is dependent on the ability of samples to resume growth after thawing (viability) and to maintain their properties identical to the cell prior to freezing, lasting to undetermined periods of time (Brand and Diller, 2004). The temperature of storage of samples after cryopreservation can have, therefore, a crucial effect in sample viability, and the maintenance regime must insure the continuously low temperatures where there is insufficient energy available in biological systems for any changes to occur (McGee and Martin, 1962).

The freezing process consists in the reduction of samples temperature at a controlled rate, usually obtained with help of controlled rate freezers (Day and Brand, 2005). The cooling rate should be slow enough to allow limited loss of intracellular water, reducing the risks of intracellular ice formation which could be deadly for samples, however also avoid the excessive loss of water, which could lead to plasmolysis and cell death (Mazur *et al.*, 1972). The process is usually facilitated by the addition of penetrating colligative cryoprotectants (CPA), which can prevent cell damage caused by volume changes and excessive solute concentration by depressing the freezing point of samples (Harding and Benson, 2013).

Several temperature points are considered critical in the process of cryopreservation: Temperature of glass transition (T_g), Temperature of homogeneous ice formation (T_h) and eutectic point (the point at which the whole system solidifies and there is no possibility of further dynamic change). Water, although mostly assumed to freeze at 0^oC, can actually supercool to well below zero and in a conventional protocol may reach around -12^oC (Fleck *et al.*, 1999). In most biological systems T_h is ca. -40^oC. And it is theorized that biological time

virtually stops below -130^{o}C and cultures maintained at LN_2 temperatures are theoretically viable for an order of 10^3 years (Mazur, 1984). This is related to another critical temperature during cryopreservation of samples, the eutectic point (Benson, 2008).

Timelines of biological resources cryobanking are now extending towards significant periods (>30-40 years) and the few examples of longevity in a wide range of biological resources suggest viability loss is not a problem for a great range of organisms, including microalgae, stored for over 20 years in LN_2 (Stacey and Day, 2007). However, the actual timescale for changes in viability has not been completely addressed, and, albeit limited, molecular mobility was demonstrated to still happen even at freezing temperatures (Walters, 2004), which could cause a continual loss of viability with time (Taylor and Fletcher, 1998; Walters *et al.*, 2004). As the rates of most chemical reactions decrease exponentially with decreasing temperatures, differences are expected in metabolic activity between cells stored at −20^{o}C and at −100^{o}C (Taylor and Fletcher, 1998). This will affect the survival of strains maintained at those temperatures, and it is to be expected that the lower the storage temperature, the greater will be the longevity of frozen cells.

Plus, temperature fluctuations during storage have the potential to induce cryoinjury and loss of viability over time (Day and Fleck, 2015). The use of low temperatures of at least -139 ^{o}C is suggested to prevent cell deterioration, as those temperatures can offer a security against temperature variations which occur, mostly, during the handling of material to include or remove samples from storage (Morris, 1981; Day and Brand, 2005). Although there are some successful reports on the storage of samples at suboptimal temperatures higher than LN_2 (Taylor and Fletcher, 1998; Day, 2013), it is expected that the longevity of these cultures would be severely reduced, although the actual timescale is still not clear. This study focused in trying to elucidate timescales for maintenance of viability of four cryopreserved microalgae strains stored at different low temperatures of storage for up to one year, as well as an analysis on the up to 40 year longevity of samples maintained immersed in LN_2 in an established microalgae cryobank, the Culture Collection of Algae and Protozoa (CCAP, UK).

4.3 Material and Methods

4.3.1 Storage of samples at different temperatures

4.3.1.1 Organisms and culture conditions

All strains used for the experiment of storage at different temperatures were from the Culture Collection of Freshwater Microalgae (CCMA – UFSCar), which is maintained at the Botany Department of Universidade Federal de São Carlos, São Paulo, Brazil (WDCM 835). *Desmodesmus communis* Hegewald (CCMA-UFSCar 030), *Ankistrodesmus fusiformis* Corda ex Korshikov (CCMA-UFSCar 333), *Kirchneriella pseudoaperta* Komárek (CCMA-UFSCar 346) and *Pseudokirchneriella elongata* Hindak (CCMA-UFSCar 498) were isolated from samples collected in subtropical ponds or reservoirs in São Paulo State, Brazil, and are currently maintained by routine serial subculture in axenic conditions, growing in WC medium (Guilard and Lorenzen, 1972) under 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark : light cycle and at a temperature of 23 ± 1°C. Tests to check for bacterial contamination in the cultures were performed regularly with WC medium modified by the addition of glucose and peptone (250 $mg.L^{-1}$ each).

4.3.1.2 Cryopreservation and Storage at different temperatures

Cultures were cryopreserved using a conventional two-step protocol with a controlled rate freezing step (Day and Brand, 2005). Aliquots of 0.5 mL of samples were aseptically transferred to 1 mL cryovals (Greiner Bio-One, Germany), where, according to treatment, 0.5 mL of either 10% (v/v) dimethylsulfoxide (DMSO) solution, or 10% Methanol (v/v) solution was added, in order to obtain final concentration of 5% of CPA for cryoprotection of frozen samples. After 15 min, samples were transferred to a controlled rate cooler (Kryo 360 1.7, Planer PLC, UK) and subjected to a cooling rate of -1°C min^{-1} from 20°C until -40°C, held at this temperature for 15 min, and then plunged directly in liquid nitrogen. After freezing, samples from each species were divided into three different groups

of 12-cryovials, which were maintained at different end temperatures: convention freezer (≈-20^{o}C) (FE-26, Electrolux, Brazil), -80^{o}C freezer (Revco Value Series, Thermo Scientific, USA) or immersed in liquid nitrogen (-196^{o}C) in a LN_2 dewar (SC-47, Sempercrio, Brazil). Triplicates were taken out from each container after storage for periods of 1 day, 30 days, 6 months and over 1 year and regrown for viability analysis.

4.3.1.3 Recovery Protocol

At each sampling point, three cryovials of each strain were recovered from each maintenance temperature and used for viability analysis. Thawing was carried out by immersing the vials in a preheated 40^{o}C water bath until all visible ice had melted (Day and Stacey, 2007). Immediately after thawing, 0.5 mL of the samples was aseptically inoculated into test tubes containing 10 mL sterile WC medium to dilute the possibly toxic concentration of the CPA used for cryopreservation. Cultures were maintained in the dark for 24h at a temperature of 23 ± 1^{o}C to prevent light-induced stress/ metabolic uncoupling. Later, samples were incubated under 100 µmol m^{-2} s^{-1} (photosynthetically active radiation), with a 12:12h dark:light cycle and at a temperature of 23 ± 1^{o}C for regrowth. Control cultures were grown in similar conditions, and assembled by inoculating 0.25 mL of non-cryopreserved samples and 0.25 mL of sterile medium in test tubes containing 10 mL of sterile medium, in order to reach the same initial concentration of cells (ca. 10^5 cells mL^{-1}) for all treatments and controls.

4.3.1.4 Viability assay

The viability of samples after freezing and storage was assessed indirectly, with analysis of the growth rate of the cultures obtained after recovery of samples. For this, the raw fluorescence of chlorophyll-*a* was quantified on a Trilogy Laboratory Fluoremeter (Turner Designs, USA) equipped with a chlorophyll *in vivo* Module (7200-043). Measurements were performed on triplicate initial cultures (immediately after inoculation) and, after 2-3 weeks, in the recovery culture tubes of each treatment and control cultures.

For comparisons between treatments, growth rate for cultures was calculated according with the equation (Wood *et al.*, 2005):

$$r = \frac{\ln (N_t/N_0)}{\Delta t}$$

where: N_0 is the initial raw fluorescence of cultures when assembled (RFU), N_t is the final fluorescence of each replicate (RFU) and Δt was the time measured in days.

4.3.1.5 Statistical analysis

Comparisons between each treatment, which were defined by the combination of one CPA and one storage temperature, and control were performed using a non-parametric ANOVA (Kruskal-Wallis). When these differences were significant ($p<0.05$), t-tests were used to compare each sample with control, to determine the periods when significant differences occurred.

4.3.2 Long term viability of samples maintained at the CCAP cryostore

4.3.2.1 Cultures tested for longevity of cryogenically stored samples

The strains used for this assay were from the Culture Collection of Algae and Protozoa (CCAP, Oban, UK) and were chosen from samples maintained in LN_2 storage at the collection cryostore for periods between 20-40 years. The species, accession numbers, time of storage, and cultivation medium are listed on Table 4.1.

Table 4.1. Characteristics of the strains analyzed after long term storage at the CCAP cryostore.

Species	Accession Number	Years of Storage	Culture medium*	T (°C) culture**	CPA
Scenedesmus obliquus	276/10	22	EG:JM	15	DMSO 5%
Chlorella zofingiensis	211/14	23	EG:JM	15	DMSO 5%
Chlorella vulgaris	211/63	23	EG:JM	15	DMSO 5%
Tetracystis intermedium	181/10	22	EG:JM	15	DMSO 5%
Tetracystis texensis	181/8	22	EG:JM	15	DMSO 5%
Desmodesmus armatus var. *brevicaudatus*	276/4E	39	PP	25	DMSO 5%
Ankistrodesmus angustus	202/4C	39	PP	25	DMSO 5%
Ankistrodesmus angustus	202/4E	39	PP	25	DMSO 5%
Ankistrodesmus braunii	202/7B	39	PP	25	DMSO 5%
Characium starrii	209/1B	39	PP	20	DMSO 5%
Spongiochloris excentrica	280/1	39	PP	20	DMSO 5%

* For medium, PP = Proteose peptone medium and EG:JM = Euglena gracilis medium:Jaworski's medium, see details on Tompkins et al. (1995); ** T(°C) is the growth temperature of cultures prior and after cryopreservation.

The current CCAP frozen collection consists of four *Working* storage cryostats, each capable of maintaining 6000 samples, and a *Master/Backup* cryostat, with the same specifications as the other storage dewars, where a reduced number of samples from each cryopreserved strain are maintained in more controlled conditions. The maintenance of samples in separate locations is a recommended process, as it avoids the loss of all current samples in case of unpredictable events which could affect the viability of samples. Temperature variations are higher on the *Working* cryostat, when compared to the *Master/Backup* cryostat, related to constant handling of samples from close places of maintenance. Also, the 40 year history of the CCAP cryopreserved collection includes at least one event of LN_2 depletion in the main *Working* cryostat whose effects are still not thoroughly studied. Thus, in this study, the viability of three samples was analyzed for each strain: two were taken from the *Working* cryostat and one from the *Master/Backup* dewar.

All samples for each strain were tracked back to the same cryopreservation batch, considering the characteristics of samples and year of storage.

4.3.2.2 Thawing and viability of samples

Samples were removed from the respective dewars and transferred in LN_2 from the cryostorage facility to the laboratory. They were thawed by immersing the vials in a 40^{o}C water bath until all visible ice had melted. Immediately after thawing, samples were aseptically transferred to conical flasks containing 9 mL of sterile medium to dilute the possibly toxic concentration of the CPA used for cryopreservation. These flasks were maintained in the dark for a 24h period and then, grown in similar conditions than pre-cryopreservation cultures. For viability analysis, the methods employed were colony formation in agar plates (Morris, 1976) and liquid culture regeneration. Thus, for each cryovial thawed, three replicates of agar plates and one liquid culture were assembled, and the data obtained was employed for comparisons with available recorded data from the time of cryopreservation. Also, this experimental design permitted an analysis of the viability levels of samples maintained on a more secure and constant environment (*Master/Backup*) and those maintained in a more variable temperature, with more handling (*Working*).

4.4 Results

4.4.1 Storage of samples at different temperatures

Generally, the variability on the growth rates of cultures after preservation was high, however, significant differences were observed between control cultures and some treatments after storage (Fig. 4.1). For samples of all strains maintained at -20^{o}C, loss of viability was observed and they failed to regrow cultures after 30 days of storage (Fig. 4.1). For *K. pseudoaperta* and *A. fusiformis* the reduction of viability was observed after only a day in storage in a conventional freezer. The storage of samples at -80^{o}C had no significant ($p>0.05$) influence on viability of samples in the first 30 days of storage. However, for samples frozen with methanol as CPA, after 6 months a severe reduction on viability was observed for *D. communis* and the other strains showed no regrowth of samples, which was confirmed after 1 year storage, when none of those samples presented regrowth (Fig. 4.1). However, samples stored at the same temperature, but cryopreserved using DMSO had no significant reduction of viability after one year storage (Fig. 4.1). Furthermore, no changes in viability were observed for samples maintained immersed in LN_2, independently of cryopreservation additive or the strain tested.

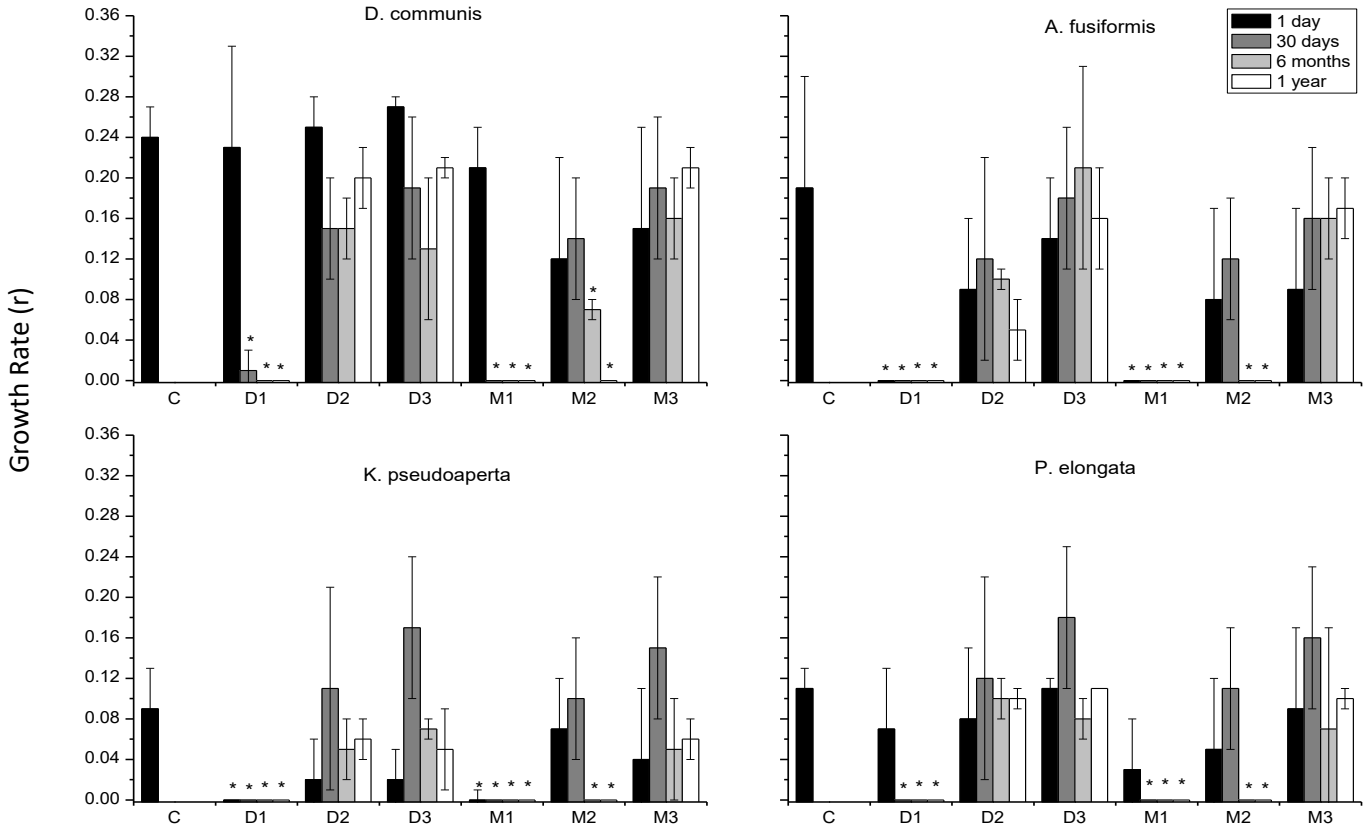

Figure 4.1. Growth rates of control (C) and samples cryopreserved using (D) dimethylsulfoxide or (M) methanol as cryoprotective additive, and maintained in storage at (1) -20°C, (2) -80°C, or (3) -196°C for 1 day, 30 days, 6 months and 1 year periods. (data shown as mean ± SD, n=3). * Significantly different to control (p<0.01)

When the CPA factor was isolated, independently of the strains tested (Fig. 4.2), similar patterns were observed for samples maintained at -20°C for both treatments, with loss of all samples after 1 month storage. The same effect was observed for samples stored at -196°C, when the viability of samples was maintained over time, independently of the CPA used in the freezing protection. However, the CPA had a crucial effect for the storage of samples at -80°C: a severe reduction on viability was observed after 6 months for samples cryopreserved using methanol as CPA which resulted in the complete loss of the ability to regrow cultures after 1 year, while the samples cryopreserved using dimethyl sulfoxide (DMSO) had no significant ($p>0.05$) loss of viability.

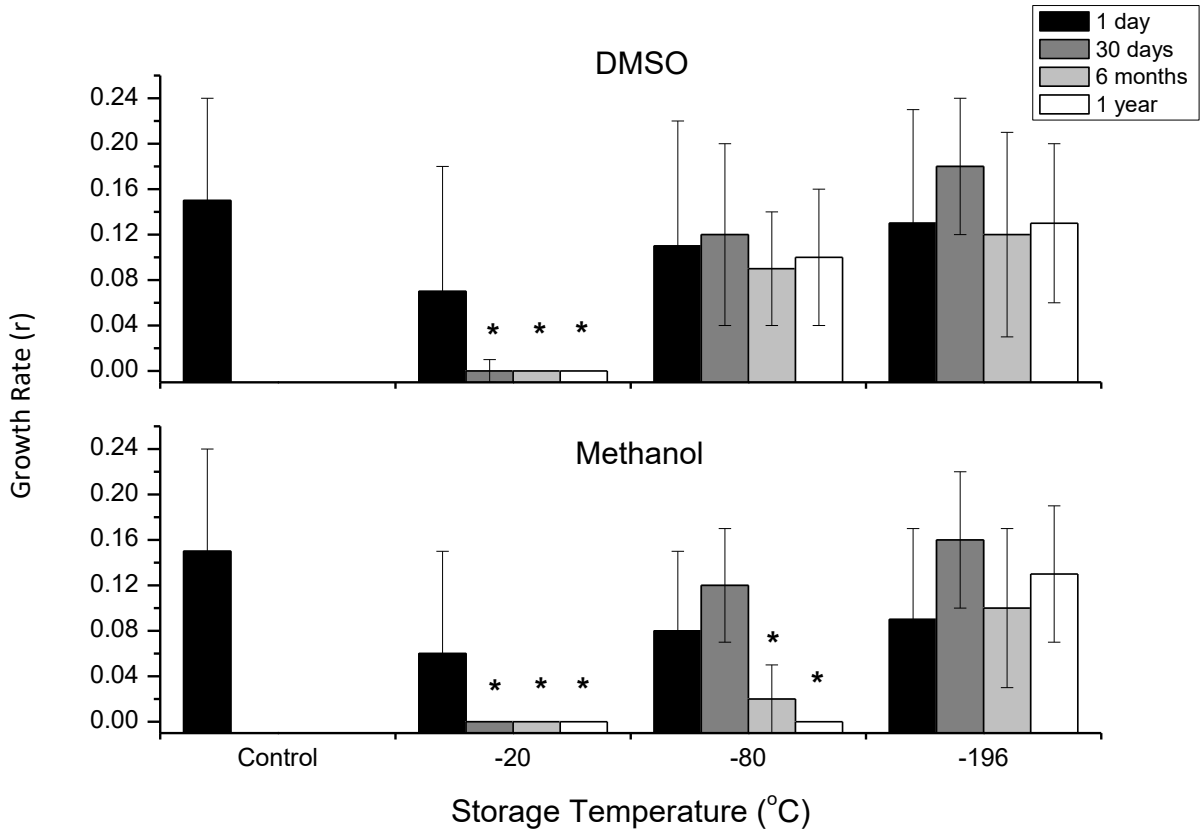

Figure 4.2. Effect of the temperature of storage on the growth rates of samples frozen with different cryoprotective additives (A) dimethylsulfoxide (DMSO) or (B) Methanol. *Significantly different to control ($p<0.01$)

4.4.2 Long term viability of samples maintained at the CCAP cryostore

Analysis of the viability of samples recovered after 20-40 years storage in liquid nitrogen (Fig. 4.3 and Table 4.2) demonstrated, in general, the maintenance of viability of samples, with recovery of robust cultures from almost all samples, the only main exception being *Characium starrii* samples recovered from the *Working* cryostat.

Table 4.2. Viability of samples after long term LN_2 storage at the CCAP collection in both the *Working* and *Master/Backup* cryostats.

			Viability in Pour Plates		Growth in Dilution Vials		
CCAP	Species	Years of Storage	*Working*	*Backup*	*Working*	*Backup*	Database info
276/10	***Scenedesmus obliquus***	22	×	✓	✓	✓	✓
211/14	***Chlorella zofingiensis***	23	✓	✓	✓	✓	✓
211/63	***Chlorella vulgaris***	23	×	✓	✓	✓	✓
181/10	***Tetracystis intermedium***	22	×	✓	✓	✓	✓
181/8	***Tetracystis texensis***	22	✓	✓	✓	✓	✓
276/4E	***Desmodesmus armatus* var. *brevicaudatus***	39	×	✓	✓	✓	✓
202/4C	***Ankistrodesmus angustus***	39	×	×	✓	✓	✓
202/4E	***Ankistrodesmus angustus***	39	×	×	✓	✓	✓
202/7B	***Ankistrodesmus braunii***	39	×	✓	✓	✓	✓
209/1B	***Characium starrii***	39	×	×	×	✓	✓
280/1	***Spongiochloris excentrica***	39	✓	✓	✓	✓	✓

Observed: ✓= growth of cultures/colonies; × = no growth;

The quantitative analysis of viability of samples (Fig. 4.3), i.e. the colony counts, could not effectively quantify viability for all samples tested. A few strains, although demonstrating reasonable growth in liquid cultures, were not able to form visible colonies on agar plates (Table 4.2), which is a more restrictive cultivation medium, as it was verified to *A. angustus* strains. However, when data were available, comparisons between viability at different points of storage were performed, and the viability percentages were similar between the recorded data available at the CCAP database and the recently obtained information from

samples recovered from the *Master/Backup* cryostat, illustrating the consistency of the maintenance at LN_2 for these samples.

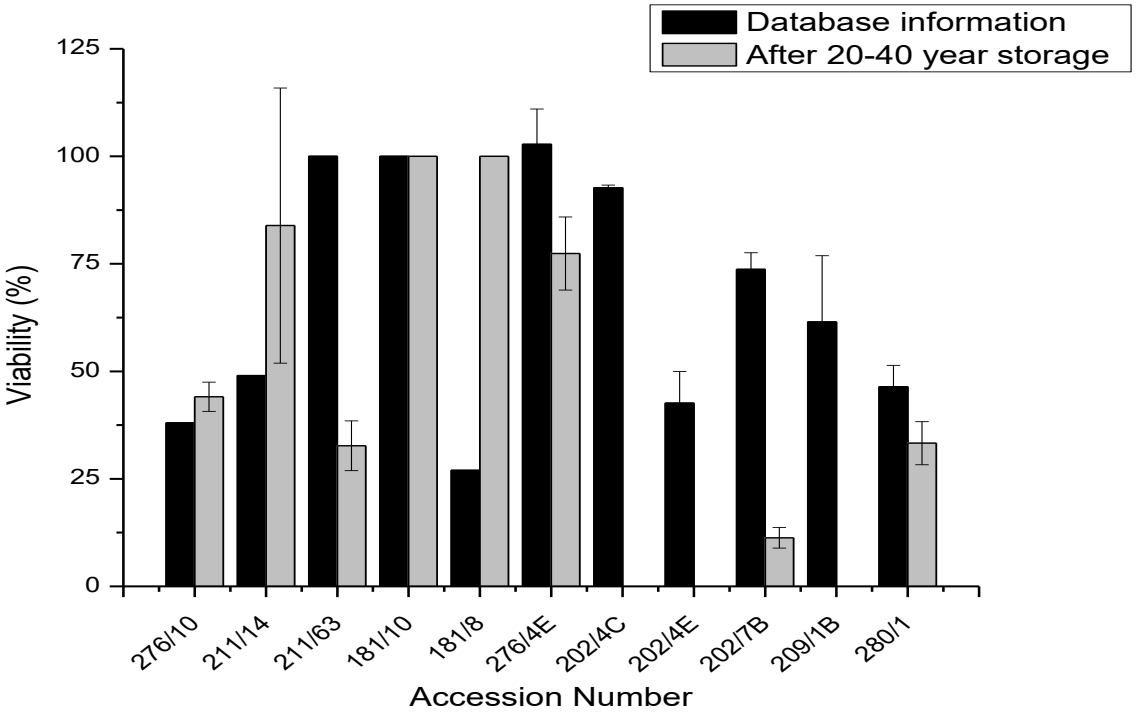

Figure 4.3. Viability (%) of samples, analyzed using colony growth in agar plates, showing a comparison between the available information from the collection database and quantitative data obtained using colony counts after recovery of samples.

Temperature variations were higher on the *Working* cryostat, when compared to the more stable *Master/Backup* cryostat, due to more often handling of samples and the nitrogen depletion event. Therefore, quantitative analysis of samples stored under both these regimes (Fig. 4.4) demonstrated differences in their ability to grow in more selective medium, as only two strains recovered from the *Working* cryostat had similar colony growth to samples maintained at the *Backup/Master* cryostat, namely the *C. zofingiensis* and *T. texensis.* However, most samples from both sources were still able to form viable cultures in liquid medium (Table 4.2).

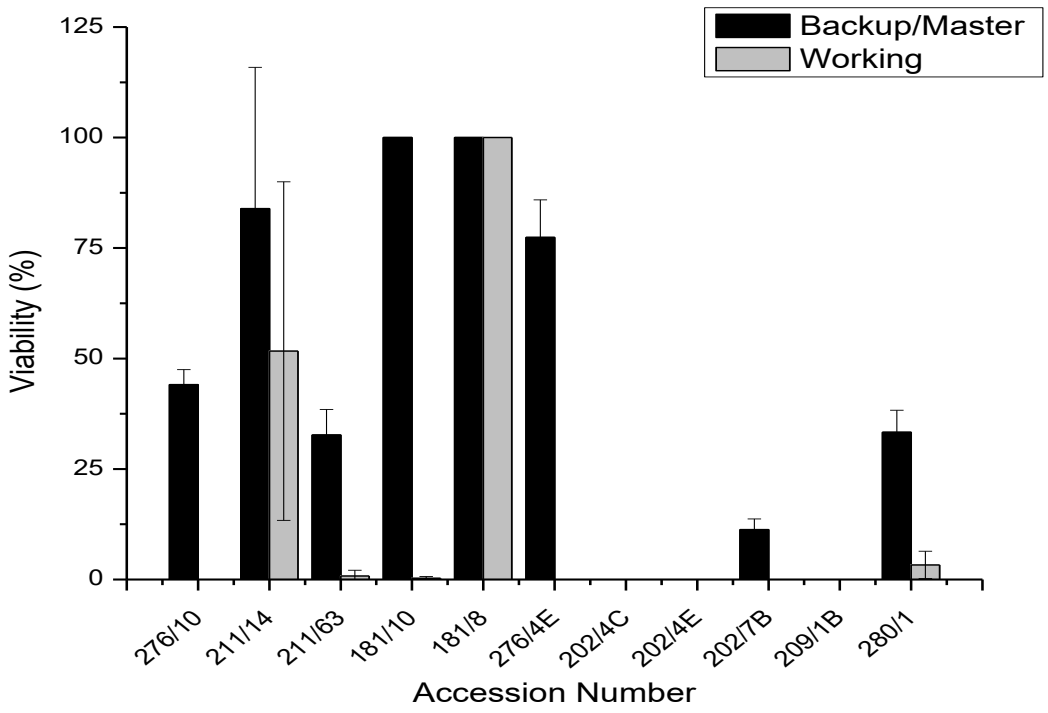

Figure 4.4. Comparison of viability (%), obtained by colony counts, between samples from the same strain maintained in constant LN_2 storage (*Master/Backup)* and in environment with known event of temperature variability (*Working*).

4.5 Discussion

The temperature of storage of frozen samples had a direct effect on their longevity. For microalgae, as is the case for most biological samples, it is advised that the storage temperature should be at least -130^{o}C, at which point all vital functions of cells should be "paralyzed" (Morris, 1981). At higher storage temperatures, biochemical and biophysical processes could still occur, causing a progressive reduction of cell recovery with increasing time of storage (Morris, 1981). When storing cryopreserved seeds, Walters et al. (2004) found that lowering the storage temperature progressively increased the longevity of the seeds. In this study, the timeline observed for viability loss in the four microalgae strains analyzed for one year showed that the deterioration of frozen cultures happened in periods of days for samples maintained at -20^{o}C and months for the replicates maintained at -80^{o}C, although only the ability of cells to form new cultures were analyzed in this study, these results were able to demonstrate a severely reduced viability of samples over time.

For microalgae, previous reports found that samples with only momentary exposure to end temperatures ranging from -10°C to -70°C, had reported viabilities up to 100% (Holm-Hansen, 1963) without use of any CPA. In their Cryoalgotox studies, Benhra *et al.* (1997) reported that *Selenastrum capricornutum* samples maintained for up to three months at -80°C had similar responses to fresh algae in toxicological test. However, other reports on the storage of algal samples at -80°C had mixed results, with limited or no success for the storage of *Chlamydomonas reinhardtii* frozen with methanol as CPA (Gresshoff, 1977; Crutchfield *et al.*, 1999), where samples lost their viability after only 24h maintenance. Similar results were found in the storage of sugarcane apices (Gonzalez-Arnao *et al.*, 1999), which presented viability loss after only 12 hour storage, and nil viability for samples after 10 and 120 days, for those maintained at -25°C and -70°C, respectively.

When considering storage of samples at sub-optimal temperatures, i.e. higher than the eutectic point, the choice of CPA and consideration of its chemical properties may have a strong effect on the longevity of samples (Fig. 4.2), and differences were already observed in an experimental period of one year. Samples protected with methanol lost most of their viability before 6 months storage when maintained at -80°C, while their counterparts which were cryoprotected with DMSO had no significant differences on their ability to divide and form new cultures. Similar results were previously reported for the storage of Cyanobacteria (Romo and Bécares, 1992), which had success only using DMSO as cryoprotective at the end temperature of -80°C.

Although both methanol and DMSO are colligative penetrating CPAs, their mechanisms of protection and physical properties are different, and therefore, samples frozen might have diverse responses to the long term storage at temperatures higher than -139°C, when biochemical and biophysical processes can still occur (Morris, 1981). For samples frozen with methanol, the storage at -80°C is over the melting point of this compound, which is -97.6°C, and this could facilitate the occurrence of molecular mobility and, therefore, cellular ageing, which can lead to viability loss. On the other hand, the melting point for DMSO is at 19°C, and although the dilution in medium can affect this characteristic for the mixture, the maintenance at -80°C is still able to provide acceptable stability for the samples which were maintained with minimal manipulation. However, for

the long term maintenance of samples, this temperature does not offer security against expected temperature variations which occur, mostly, during the handling of material to include or remove samples from storage (Day and Brand, 2005), which could be fatal to more sensitive strains of microalgae.

Any manipulation of samples frozen with methanol and stored at -80^{o}C, when considering the freezing point of this compound, even in mixtures with water, allows events of thawing and re-freezing of samples in a non-controlled environment, facilitating the process of ice formation which is one of the mains causes of cryoinjury on cells, and could lead to the loss of samples (Mazur *et al.*, 1972; Day and Fleck, 2015). The storage of samples immersed in LN_2 provided enough temperature stability in the one year experiment and no significant differences were found on the growth of control samples and samples stored for up to one year, independently of the CPA used for freezing (Fig. 4.1). As this temperature of storage is much lower than the eutectic point of any of the compounds used during the freezing process, it offers a security for the samples, avoiding any events of thawing of samples, as long as the levels of LN_2 are maintained (Benson, 2008). Furthermore, the studies with samples stored for almost 40 years at the CCAP collection provided helpful information on the maintenance of frozen cultures for long periods of time (Table 4.2; Fig. 4.3). In a previous study with samples from the CCAP cryopreserved collection, Day et al. (1997) reported the maintenance of viability on cryopreserved samples of microalgae for around 20 years immersed in liquid nitrogen. Indeed, in our studies, the qualitative analysis on the recovery of viable cultures in liquid medium showed reliable growth on most samples, which formed dense cultures of microalgae when inoculated in fresh medium. When records were available, the collection database provided basis of comparison for the quantitative analysis on the viability of samples during long term storage (Fig. 3.3) and it was demonstrated that most samples maintained in stable conditions during the stock period (*Master/Backup* cryostat) were viable and able to generate cultures, which could be directly applied as substrates for further research and applications in biotechnology.

The reliability of cryopreservation and the maintenance of samples in LN_2 was also analyzed by Nakanishi et al. (2012), who reported sustained levels of survival of cryopreserved strains for a period of 15 years with no loss of chlorophyll-a content on cells,

which was found to happen in serial subculturing of *Chlorella vulgaris.* Furthermore, reports on the genomic stability (Müller *et al.*, 2005) and biotechnological functionality (Hipkin *et al.*, 2014) of algae maintained in cryopreservation reinforce the advantages of cryopreservation of samples to maintain strains of microalgae for undetermined periods of time (Day and Stacey, 2008).

Although the storage at LN_2 offers a fairly secure environment for samples, the correct management of the nitrogen supply in the storage containers is a crucial step to avoid temperature fluctuations, which could be fatal for the more sensitive strains. The 40+ year history of the CCAP cryostore includes one major nitrogen depletion event in the *Working* cryostat before the current pattern of maintenance and quality control was established. The full consequences of this were still not completely known, although adequate regrowth of samples has been observed for samples which were restored to liquid nitrogen temperatures as soon as the problem had been recognized. When analyzing samples from the same cryopreservation batch maintained in a stable environment (*Master/Backup*) and samples that suffered more constant handling and temperature variations (*Working*) (Fig. 4.4), a severe decline on the viability of samples from the latter was observed when a more selective growth test was used for the analysis. Although most cultures were still able to form viable liquid cultures, the quantitative analysis of the growth on the selective media offered information of a profound effect of the temperature variation on the overall viability of samples, reinforcing the importance of quality control and refined management on a cryopreserved biobank. The storage temperature and CPA choice are, therefore, crucial for the maintenance of the viability of samples, and, for long term conservation of samples, unexpected temperature fluctuations should be cautiously considered and reported, as they may lead to the loss of important samples in a very short time-frame.

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5 Genetic and Phenotypic stability of cryopreserved samples

5.1 Abstract

Culture collections play a major role in underpinning research and further biotechnological application of microalgae research. Thus, they are required to offer standardized inocula to ensure the expected development and characteristics of the target cultures, maintaining its aggregated value. The use of cryopreservation for the maintenance of samples, in theory, is able to conserve the samples characteristics for an undetermined length of time, as long as thermal stability is maintained. This is a recommended method, when compared to the serial subculturing, as the latter is more sensitive to handling factors and conditions. However, the freezing and thawing process can be potentially damaging for microalgae cells, which are exposed to extreme conditions, risking physical, chemical and physiological stress. Therefore, the necessity arises for tests to ensure the maintenance of frozen culture characteristics as ´true to type´ material or if the application of a standardized cryopreservation protocol might affect the genetic and functional profiles of organisms. In this study, Amplified Fragment Length Polymorphism (AFLP) and Matrix Assisted Laser Desorption Ionization mass spectrometry (MALDI-TOF MS) were applied to assess possible differences in the responses of subcultures and their cryopreserved counterparts at molecular and physiological levels for green algae strains. In general, growth of previously cryopreserved cultures followed the same patterns, and had similar responses to exposure to high temperature stress, although *Kirchneriella pseudoaperta*, which had reported lower post-thaw viability, needed a longer recovery (lag) phase, which could also be observed on the biochemical analysis of these samples. This strain was also tested for genetic stability after repeated freezing/thawing cycles, and no differences in fragment patterns were observed from those obtained for samples pre-cryopreservation.

5.2 Introduction

Culture collections are responsible to ensure the continuity of microalgae research worldwide. However, quality standards need to be reached to insure the stability of the target organisms' characteristics, e.g. production of secondary metabolites, a key aspect for the services provided, which will guarantee the quality of strains and their recognized ecological and commercial importance (Benson, 2008; Brand *et al.*, 2013). Although serial-subculturing of metabolically active cultures has been the standard protocol for culture maintenance decades (Lorenz *et al.*, 2005), several reports can be found on the loss of the culture characteristics over time in cyanobacteria and eukaryotic microalgae (Day and Fleck, 2015). Cryopreservation, i.e. the maintenance of organisms at ultra-low temperatures, temporarily pauses the cellular metabolism, which is known to ensure that cells are stable *ad infinitum,* as long as thermal stability is maintained, usually reached with immersion in liquid nitrogen (LN) (Grout *et al.*, 1990; Day and Brand, 2005). The cryopreservation of living valuable strains would be beneficial, therefore, for their long term storage, reducing the risks of genetic changes and loss of commercial biosynthetic and molecular attributes (Benson, 2008). However, the process of cryopreservation *per se* has the potential to cause cryoinjury, exposing the organism to physical, chemical and physiological stress (Reed *et al.*, 2004; Harding *et al.*, 2010; Day and Fleck, 2015) and therefore effecting the characteristics of post-thaw cultures, which should be able to replicate the same biochemical and physiological conditions observed prior to the process (Benson, 2008). This has been a challenge particularly for the larger and morphologically complex algal strains, which are still mostly recalcitrant to the process. Genetic differences may also appear during the freezing-regrowth process, reinforcing the importance of verifying if cryopreserved samples can be considered 'true to type' material (Day *et al.*, 2010; Harding *et al.*, 2010).

Very few studies are available on the genetic and phenotypic stability of cryopreserved samples and these are mostly focused on plant cells and embryos (Fernandes *et al.*, 2008; Martín *et al.*, 2011). For microalgae, even less information can be found, and with large gaps in information on possible causes of changes (Day and Fleck, 2015) and there is still a paucity of published evidence to validate the claims that cryopreservation demonstrably guarantees both functional and genetic stability for a wider range of algae. In

this study, the effects of cryopreservation on strains of microalgae were analyzed using fingerprinting techniques based on genetic, *Amplified Fragment Length Polymorphism* (AFLP), and biochemical analysis, using Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry. The AFLP technique was first described by Vos et al (1995) as a technology for DNA fingerprinting, allowing the wide range analysis of the organism's genome, without previous sequence-knowledge, and has produced reliable patterns for comparison between cryopreserved and subcultures samples for microalgae studies, although it has been mostly applied on studies on the biodiversity of higher plants at molecular level (Müller *et al.*, 2007). Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) is a powerful tool for the proteomic analysis of microorganisms, with usual application for the rapid identification of strains (Lewis *et al.*, 2000; Murugaiyan *et al.*, 2012). This approach has been successfully employed for a proteomic based chemotaxonomy, with high reproducibility of results between samples (Emami *et al*., 2015).

These techniques were employed to obtain basis for comparison between cultures of microalgae strains which were submitted to the cryopreservation protocol and those originated only from direct subculturing from metabolically active inocula. The growth of cultures and biochemical composition of cells, analyzed using MALDI-TOF MS, was followed over time for cultures of green microalgae assembled from either cryopreserved or serial subculture inoculums. For furthering analysis on the possible risks of cryopreservation protocol for the maintenance of strain characteristics, tests were performed with microalgae samples submitted to repeated freezing and thawing cycles, and their biochemical (MALDI-TOF MS) and genetic (AFLP) integrity was evaluated. These tests are essential for the verification of genetic and phenotypic stability of cryopreserved microalgae strains and validation of the cryopreservation protocol.

5.3 Material and Methods

5.3.1 Physiological responses of cryopreserved and non-cryopreserved cultures after recovery of cultures and growth.

5.3.1.1 Organisms

The differences in the response of microalgae growth directly after recovery from cryopreservation were investigated for three strains of microalgae from the Culture Collection of Freshwater Microalgae (CCMA – UFSCar), based at the Botany Department of Universidade Federal de São Carlos, São Paulo, Brazil (WDCM 835): *Coelastrum sphaericum* (CCMA-UFSCar 060), *Monoraphidium contortum* (CCMA-UFSCar 306) and *Kirchneriella pseudoaperta* (CCMA-UFSCar 346) which are currently maintained by routine serial subculture in axenic conditions, growing in WC medium (Guilard and Lorenzen, 1972) under 100 µmol photons m^{-2} s^{-1} (photosynthetically active radiation), on a 12:12h dark : light cycle and at a temperature of 23 ± 1^{o}C. These strains were selected based on their post-thaw viability levels, previously analyzed in this thesis (Chapter 2). Tests to check for bacterial contamination in the cultures were performed regularly with WC medium modified by the addition of glucose and peptone (250 $mg.L^{-1}$ each).

5.3.1.2 Experimental design

The growth of samples was followed, for each of the tested species, in triplicate cultures assembled from cryopreserved or non-cryopreserved inoculums, in order to verify the physiological stability of cultures after possible damage, originated from the cryopreservation protocol. Non-Cryopreserved cultures were obtained directly from subculturing the starter culture, with enough volume inoculated to fresh WC medium to obtain a final concentration of $10^4.mL^{-1}$ microalgae cells. For the establishment of cryopreserved cultures, aliquots of the starter culture were treated with cryoprotective

additive (CPA) and frozen using the standard protocol, as described in Chapter 2 (Day and Brand, 2005). Samples were maintained in liquid nitrogen for 2 hours, thawed following the established protocol, and immediately diluted in sterile WC medium to obtain the experimental cultures. Similar volumes of the original inocula were used on both cryopreserved and non-cryopreserved cultures. In order to facilitate sampling and avoid contamination, the established cultures were divided into 12 identical sterile 100 mL flasks for each treatment, with final volume of 50 mL of culture each, which were incubated under controlled conditions using a Multitron Standard Incubation Shaker (Infors HT, Switzerland) equipped with photoperiod control, at 23 ^{o}C, 90 rpm; photoperiod of 12:12 hours light:dark. After the determined periods of time (5, 12, 17 and 22 days), three test cultures of each treatment were used for further analysis. Growth of samples was analyzed indirectly, employing analysis of the Optical Density of samples in a spectrophotometer (HACHI, Japan), with readings at 680 and 750nm (Griffiths *et al.*, 2011). For the biochemical analysis of samples, aliquots of each culture (15 mL) were centrifuged to obtain cell pellets, which were later treated and analyzed in a MALDI-TOF MS.

5.3.2 The effects of repeated cryopreservation cycles on the genetic and phenotypic stability of microalgae strains

5.3.2.1 Organisms

The organisms chosen for the genetic and phenotypic stability analysis after repeated freezing and thawing cycles were two strains with different responses to the cryopreservation process: *Ankistrodesmus fusiformis* (CCMA-UFSCar 333), which had high post-thaw viability after the cryopreservation protocol and *Kirchneriella pseudoaperta* (CCMA-UFSCar 346), which had demonstrated lower viability levels, although growth was still observed for this strain (Chapter 2). Cultures are maintained at CCMA-UFSCar, under the same conditions described previously (5.3.1.1).

5.3.2.2 Experimental Design

Starter cultures (initial) were prepared from metabolically active cultures, and incubated under the previously described environmental conditions for a period of 7 days. These samples were then employed for the first freezing and thawing cycle and aliquots of the cultures were separated for MALDI-TOF MS and AFLP analysis (Pre-Cryo). After seven days of regrowth of the cryopreserved cultures, samples were separated for stability analysis (F1.7) and aliquots were submitted to a new cryopreservation cycle. Sucessive cryopreservation cycles with 7 days recovery between them were applied, and samples were separated for MALDI-TOF MS and AFLP analysis (F2.7; F3.7; F4.7). The standard cryopreservation protocol was applied for each cycle (Day and Brand, 2005), using DMSO as CPA and cultures were maintained frozen for at least 3 hours before thawing and dilution in fresh sterile WC medium. The samples obtained and their respective treatments are summarized in Table 1, and for each treatment aliquots were centrifuged and cell pellets separated for Maldi-TOF MS analysis and Amplified Fragment Length Polymorphism (AFLP).

Table 5.1 - Treatments applied during tests with repeated freezing and thawing cycles

Treatment	Freezing cycles	Recovery time between cycles	Recovery for sampling
Initial (Ini)	0		7 days
F 1.7	1		7 days
F 2.7	2	7 days	7 days
F 3.7	3	7 days	7 days
F 4.7	4	7 days	7 days

(Pre-cryo = samples before cryopreservation; Fa.b = samples submitted to “a” cryopreservation cycles with “b” recovery time between them.)

5.3.3 Biochemical analysis: Maldi-TOF spectra.

The comparison on biochemical composition of microalgae cells between treatments at different points of the growth curve, of after repeated cryopreservation cycles, were determined using Matrix-Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectrometer, equipment used in association with Prof. Dr. Edson Rodrigues Filho,

Chemistry Department, Federal University of Sao Carlos. Samples preparation and analysis were as described by Mello (2016). Briefly, samples were centrifuged in order to obtain an algal pellet, which was stored at -80^{o}C until analysis. For analysis, samples were then freeze-dried, re-suspended in *α-Cyano-4-hydroxycinnamic acid* (HCCA) matrix solution, homogenized using sonication, and spotted using 2 µL of algae-matrix solution in a ground steel plate for MALDI analysis. Three spectra were obtained for each replicate, totalizing 9 spectrums for each treatment analyzed. Spectra were treated for further analysis using the software *flexAnalysis* (Bruker Daltonics, USA). Similarity and cluster analysis were performed in *Bionumerics 7.6* software (Applied Maths, Belgium). Replicates obtained for each treatment were grouped and a 90% similarity filter was applied to reach a summary spectrum for each treatment. A similarity matrix was generated from the previously described summary spectra, using curve based Pearson correlation for comparisons between treatments and sampling period.

5.3.4 Amplified Fragment Length Polymorphism (AFLP)

Genomic DNA from samples was extracted using a MOBIO PowerSoil® DNA Isolation Kit, employing 30-75 µL of concentrated algae from each sample for extraction. This kit includes physical and chemical steps to insure high-yield DNA extraction. All isolations were performed following the manufacturer´s protocol and samples were quantified on 1% agarose gel. Tubes were stored at -80^{o}C until further application. For AFLP analysis, genomic DNA was extracted twice for each replicate, on different days, to identify potential variations on patterns caused by the extraction process (Müller *et al.*, 2007).

Reactions of restriction and ligation of the genomic DNA were performed simultaneously, in a single reaction (Mannschreck *et al.*, 2002; Müller *et al.*, 2005) using two different endonucleases: *Mse*I (NEB, USA), which is a frequent cutter, and *EcoR*I (NEB, USA), which is a infrequent cutter. The protocol for incubation was adapted from Müller *et al.* (2005), modified to apply to larger volumes of genomic DNA (10 µL) in order to obtain the required concentration for the reaction, in a total volume reaction of 20 µL. Pre-selective amplifications were performed using 4 µL of restriction-ligation products, with primers *Mse*I

+ 0 and *EcoR*I + 0 (2.5 pmol each, primer sequences as in Vos *et al.*, 1995) in a total reaction volume of 20 µL. The reaction was checked in a 1.5% agarose gel and diluted 2-10 fold according with results obtained (Müller *et al.*, 2005). Selective amplification was performed using the following primer combinations: EcoRI+A (10 pmol)/MseI+C (10 pmol) and EcoRI+C (5 pmol)/MseI+C (10 pmol), in a final volume reaction of 20 µL. EcoRI+A was labeled with the fluorochrome 6-FAM and EcoRI+C with HEX (Sigma). The reproducibility of the AFLP patterns was confirmed by three replicate reactions for each sample, one reaction using the first DNA extraction and two using the second DNA extraction (Müller *et al.*, 2005).

The fluorescently labeled AFLP fragments were separated by capillary electrophoresis, in a service provided by *Macrogen Inc*., using a 400HD ROX Internal Standard Size Marker (Applied Biosystems). Fragment sizing was performed using Peak Scanner 2 (Applied Biosystems, USA) and the following fingerprinting analysis was performed using the *BioNumerics 7.6* software (Applied Maths, Belgium).

5.4 Results

5.4.1 Physiological responses to the cryopreservation process: culture growth

Cryopreserved inoculums from *Coelastrum sphaericum* (CCMA-UFSCar 060), *Monoraphidium contortum* (CCMA-UFSCar 306) and *Kirchneriella pseudoaperta* (CCMA-UFSCar 346) were able to grow and establish robust cultures (Fig. 5.1). The cryopreserved samples of *K. pseudoaperta* demonstrated a longer lag phase on incubation at 23^{o}C (Fig. 5.1); however, normal growth was still observed after this period, reaching high concentration of cells, which were comparable to the cultures established from non-cryopreserved samples.

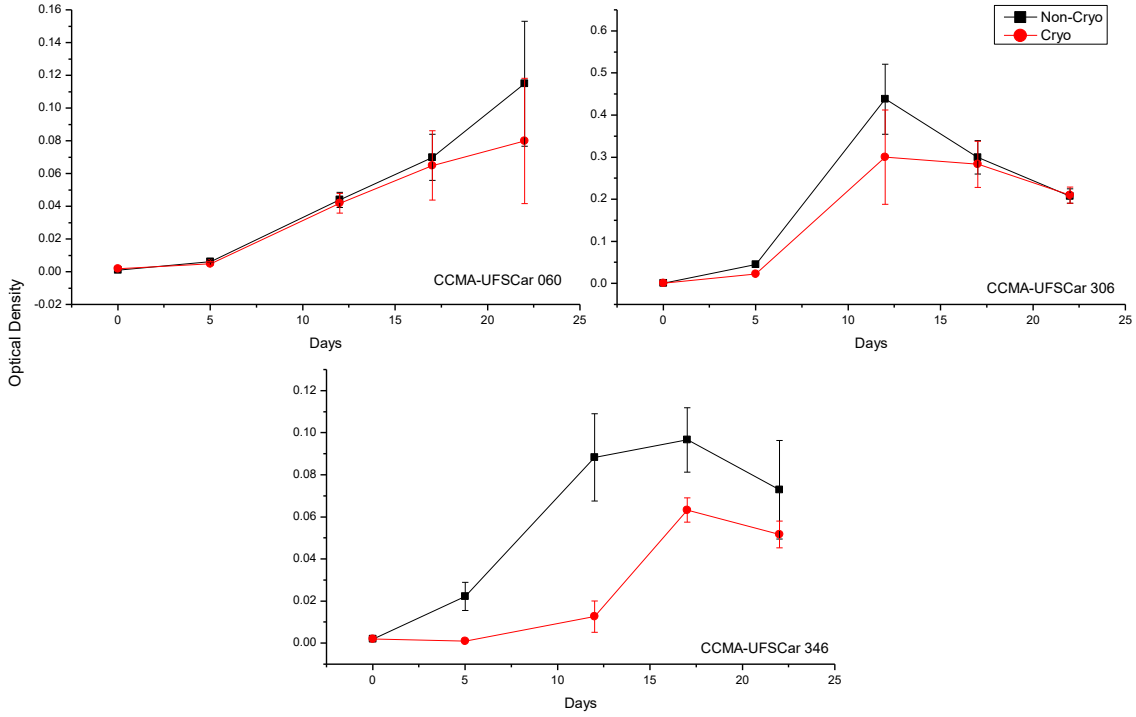

Figure 5.1 - Growth of *Coelastrum sphaericum* (CCMA-UFSCar 060), *Monoraphidium contortum* (CCMA-UFSCar 306) and *Kirchneriella pseudoaperta* (CCMA-UFSCar 346), inoculated from cryopreserved samples (red) and non-cryopreserved control cultures (Black), incubated for growth at 23°C .

5.4.2 Culture growth and biochemical profile of cryopreserved and non-cryopreserved cultures of microalgae using Maldi-ToF analysis.

Biochemical profiles of *K. pseudoaperta* (CCMA-UFSCar 346) samples from cryopreserved and non-cryopreserved cultures after growth were analyzed using Maldi-TOF MS, and exemplar spectra of each treatment are presented in (Fig. 5.2). The visual analysis of the protein profiles for these strains provide some information explaining the distances obtained between the treatments analyzed of these samples (Fig. 5.3). However, it must be considered that in some samples the baseline noise was higher, which could have affected the peak detection (e.g. Fig. 5.2E).

The analysis of similarity between the spectra obtained at different points during the growth of *K. pseudoaperta* indicated that overall, although differences were observed following the culture growth, similarities were observed in the proteome analysis between treatments cryopreserved and non-cryopreserved (Fig. 5.3). However, the delay observed in the development of cryopreserved cultures (Fig. 5.1) when compared to the control non-cryopreserved cultures, also had consequences for the biochemical analysis, as higher matches were observed between different sampling points (for example, Cryo 17 was highly matched to Ncryo 12). The largest levels of dissimilarity were observed between the extreme sampling points, although this were possibly related to the physiological status of cultures and/or the acquisition of spectrums, and not related to consequences of the cryopreservation process.

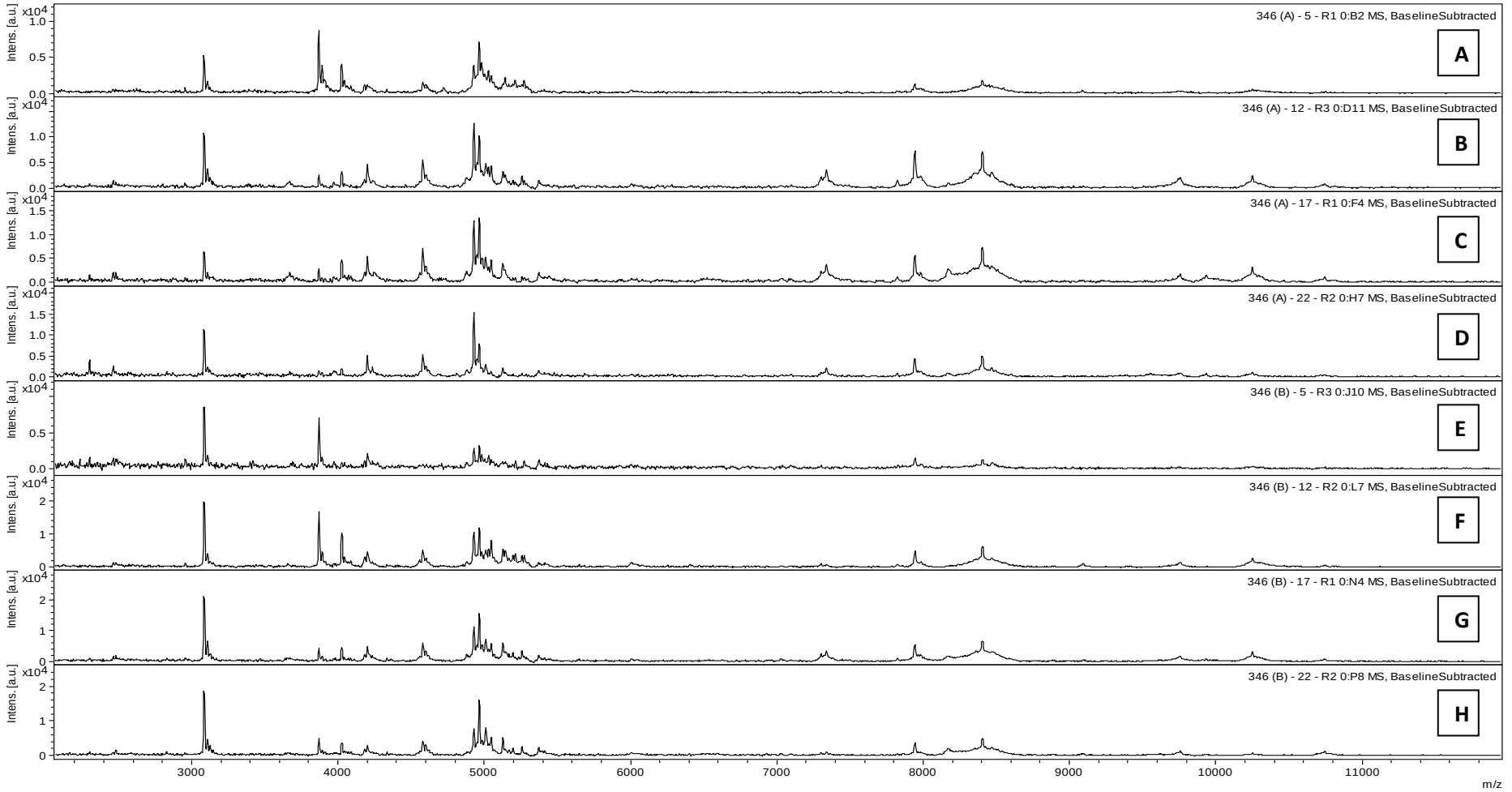

Figure 5.2 – Maldi-TOF MS profiles of non-cryopreserved (A, B, C, D) and recovered cryopreserved (E, F, G, H) *K. pseudoaperta* cultures. A,E = after 5 days; B, F = after 12 days; C,G = after 17 days; D, H = after 22 days.

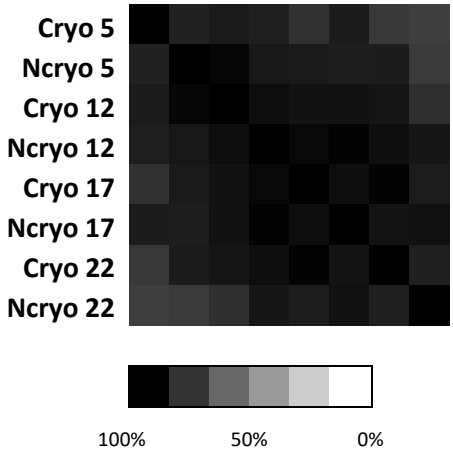

Figure 5.3 – Similarity matrix between MALDI-TOF spectra obtained for *K. pseudoaperta* (CCMA-UFSCar 346), using Pearson correlation for similarity analysis. Ncryo = samples not cryopreserved; Cryo = cryopreserved samples ; numbers are days incubation at 23°C after treatment.

Both the cryopreserved and non-cryopreserved cultures of *M. contortum* had highly similar spectra at all sampling points during the growth experiment (Fig. 5.4 and Fig. 5.5). It was noted that the recovery of this strain was faster, when compared to *K. pseudoaperta*, and the largest dissimilarities were observed, for both treatments, between the samples from day 22 of the culture and the previous samples. However, these difference were assumed to be associated with the natural development of the microalgal culture, entering stationary phase, and not consequences of the cryopreservation process.

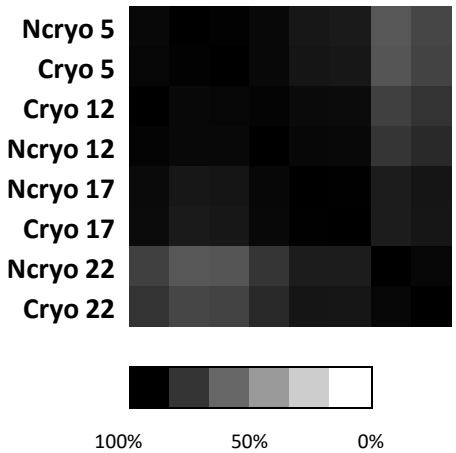

Figure 5.4 – Similarity matrix between MALDI-TOF spectra obtained for *M. contortum* (CCMA-UFSCar 306), using Pearson correlation for similarity analysis. Ncryo = samples not cryopreserved; Cryo = cryopreserved samples; numbers are days incubation at 23°C after treatment.

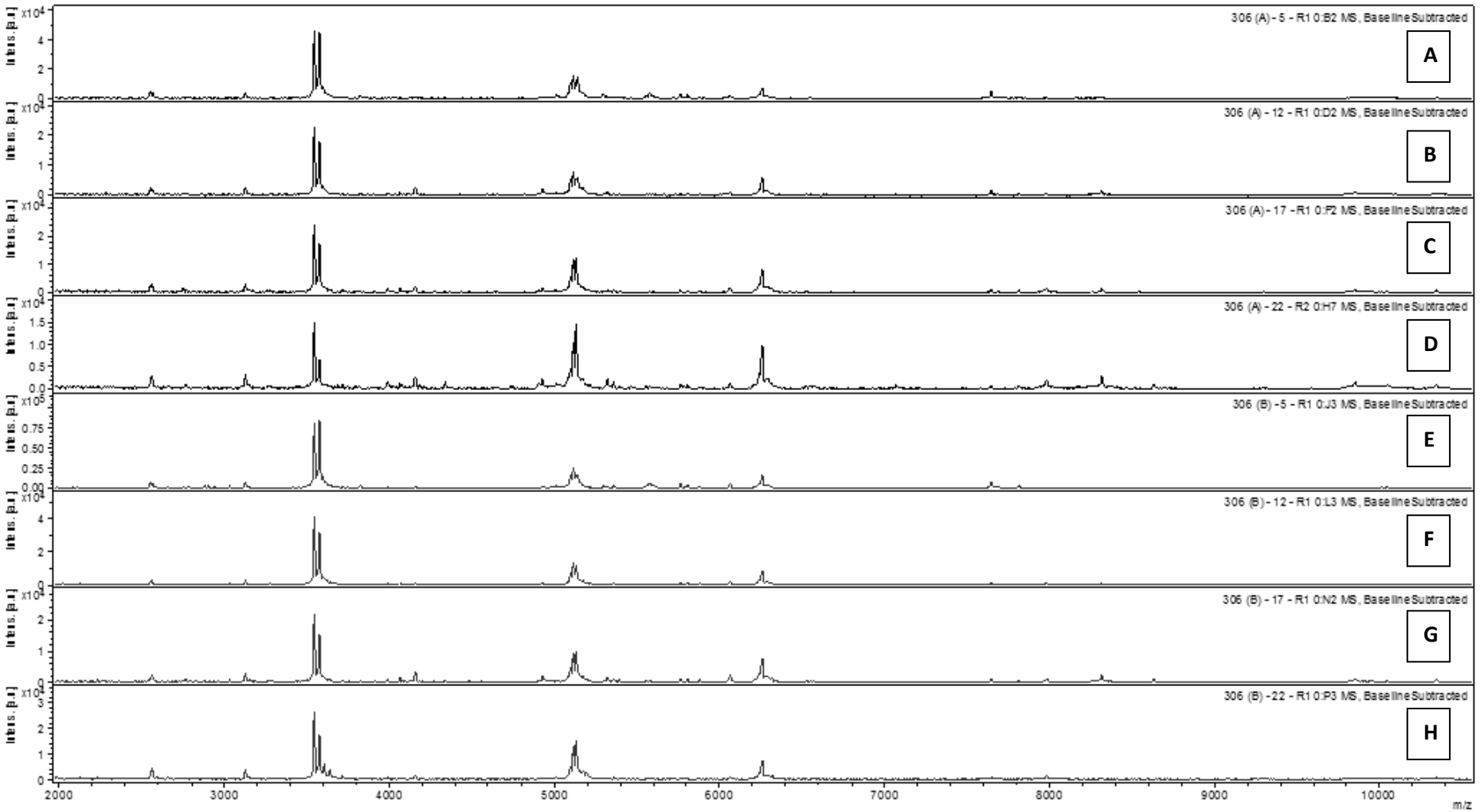

Figure 5.5 –Maldi-TOF MS profiles of non-cryopreserved (A, B, C, D) and recovered cryopreserved (E, F, G, H) *M. contortum* cultures. A,E = after 5 days; B, F = after 12 days; C,G = after 17 days; D, H = after 22 days.

5.4.3 Genetic and biochemical (Maldi-TOF) profiles of microalgae submitted to repeated cryopreservation cycles.

Overall, the analysis of *Ankistrodesmus fusiformis* after several repetitions of the freezing and thawing protocol showed highly similar MALDI-TOF profiles (Fig. 5.6) between treatments, which were also directly comparable to samples before cryopreservation (Pre-Cryo). The analysis applied for similarity comparison considered only the presence/absence of peaks, and the number of different peaks was used as index for this analysis, to avoid bias on data related to sampling and preparation for MALDi-TOF analysis. Unfortunately, the genetic analysis using AFLP for these samples was not determined, as the genomic DNA obtained from extraction was not responsive to restriction/ligation reactions. Several adaptations on the protocol were tried, without positive response to date.

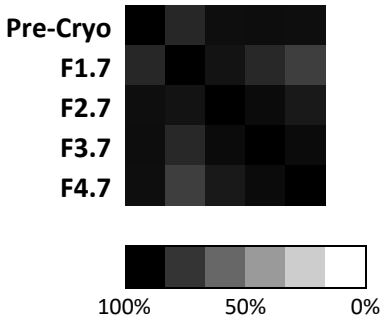

Figure 5.6 – Similarity matrix of MALDI-TOF spectra for *A. fusiforms* (CCMA-UFSCar 333), using the number of different peaks for similarity index. Pre-cryo = samples before cryopreservation; Fa.b = samples submitted to "a" cryopreservation cycles with "b" recovery time between them.

Although slightly larger differences were observed on analysis of MALDI-TOF profiles of *K. pseudoaperta* (Fig. 5.7), overall, the similarity of these samples were still comparable to similarities between replicates of the same culture, and not a consequence of permanent damage to cell functionality, induced by the cryopreservation protocol.

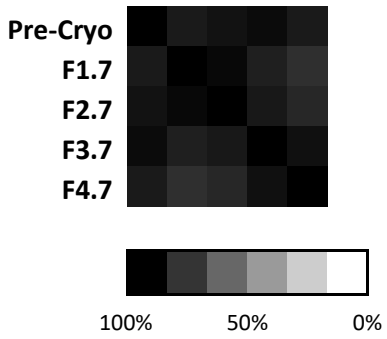

Figure 5.7 – Similarity matrix between MALDI-TOF spectra for *K. pseudoaperta* (CCMA-UFSCar 346), using the number of different peaks for similarity index. Pre-cryo = samples before cryopreservation; Fa.b = samples submitted to "a" cryopreservation cycles with "b" recovery time between them.

The analysis of *K. pseudoaperta* samples, performed using AFLP patterns, demonstrated that there was high reproducibility of peak profiles in each treatment (cryopreservation cycles). The fragment size profiles obtained for samples analyzed after each cryopreservation cycle and the samples prior to cryopreservations had high similarity (Fig 5.8)..

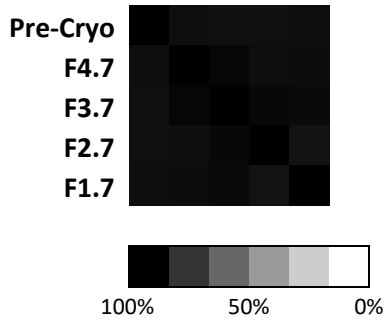

Figure 5.8 – Similarity matrix of AFLP patterns from replicates of *K. pseudoaperta* (CCMA-UFSCar 346) treatments, using the Jaccard index in a binary matrix. Pre-cryo = samples before cryopreservation; Fa.b = samples submitted to "a" cryopreservation cycles with "b" recovery time between them.

5.5 Discussion

The successful storage of organisms over long periods of time requires the maintenance of samples within quality standards, to ensure the continuity of research and production of reproducible results (Tedeschi and De Paoli, 2011). Although minimum levels

of viability were historically considered critical to the conservation and maintenance of genetically stable algal biological resources (Morris, 1981), further research on microalgae strains found no correlation between the viability levels and the genetic consistency of post-thaw samples (Müller *et al.*, 2007). However, the post-thaw viability may work as mirrors for the susceptibility of samples to the selection of cryo-tolerant populations within samples, which may have a different (and undesirable) set of characteristics than the pre-cryopreservation cultures (Day and Fleck, 2015).

Application of sub-optimal protocols for cryopreservation might differently affect closely related cultures (Day and Brand, 2005) and usually, the existence of a longer lag period after thawing indicates the occurrence of intracellular damage, which may also affect the cell genome (Chetverikova, 2011). Indeed, for the samples tested in this project, only the growth of *K. pseudoaperta* was affected, and these changes had some small effects on the biochemical profiles of samples. For *M. contortum* and *C. sphaericum*, the growth curves obtained were mostly similar between non-cryopreserved and cryopreserved initiated cultures. These growth patterns were also reflective of the viability rates, previously obtained for these cultures (Chapter 2). However, the cryopreservation process might have affected the sensitivity of this strain to environmental fluctuations, which are not detectable on optimal growth conditions, which is currently the subject of ongoing further analysis.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) is a powerful tool for the analysis of proteins, with usual application for the rapid identification of microorganisms (Lewis *et al.*, 2000; Murugaiyan *et al.*, 2012). This approach has been successfully employed for a proteomic based chemotaxonomy to discriminate between closely related strains of *Dunaliella* (Emami *et al.*, 2014) and similar studies have been reported for other microalgae groups (Barbano *et al.*, 2015; Lee *et al.*, 2015; Mello, 2016). In this project, MALDI-TOF spectra were obtained before and after algae were subjected to cryopreservation and protein profiles were analyzed to provide an overview on the possible physiological responses to the freezing and thawing stress. Overall, the analysis of the samples growth at 23^{o}C demonstrated that biochemical profiles were influenced by the phase of culture growth, while the differences between the cryopreserved and non-cryopreserved treatments were minimal. However, it was necessary to take into

consideration the delay in recovery post-thaw of *K. pseudoaperta* when making the comparisons.

The physiological and functional stability of cryopreserved algal samples has previously been reported by Hedoin et al (Hédoin *et al.*, 2006), in tests with the biotechnologically important algal strains *Porphyridium cruentum* and *Planktothrix,* which are producers of Zeaxanthin and beta-carotene, and bioactive cytotoxins, respectively. These algae were successfully cryopreserved and productivity levels of these compounds were maintained unchanged from standard and post-thaw inocula. Similarly, in a study on transgenic diatom *Thalassiosira pseudonana,* Hipkin et al. (Hipkin *et al.*, 2014) observed high levels of post-thaw viability with no reduction in expression of the inserted gene, as well as maintenance of the functionality of the protein. Although no specific compounds were identified or isolated for the presented analysis, the patterns obtained for culture replicates within the same treatment had high similarity, providing an interesting tool for the analysis of stability of cryopreserved samples.

Very little experimental information is available on genetic stability of samples maintained in culture collections. The amplified fragment length polymorphism (AFLP) method was employed by Vos et al (1995) as a technique for DNA fingerprinting employing the selective amplification of digested DNA fragments to generate and compare unique patterns, without the necessary prior knowledge on the targeted genome (Paun and Schönswetter, 2012). Samples of *K. pseudoaperta,* which were exposed to repeated cryopreservation cycles, were analyzed in this study, generating a similarity matrix between treatments. All samples demonstrated similar electropherograms, with similarity comparable to replicates of the same treatment (over 95%). Also, no detectable alterations on phenotypic expression, as visualized on protein analysis by MALDI-TOF MS were observed on analysis of the samples derived from these treatments, and the similarity values obtained between treatments were comparable to similarities between replicates of the same sample (similarity over 80%). In a study with isolates of *Chlorella vulgaris,* the AFLP profiles of strains of the same isolate that were maintained by continuous sub-culturing over many decades had no genomic differences to those obtained for samples stored in cryopreservation for up to 20 years (Müller *et al.*, 2005). However, in a study with AFLP patterns generated from 28

species of microalgae from samples before and after cryopreservation processing, small, or multiple alterations of DNA fragments of 14 strains of the strains tested were observed, although these did not appear to correlate with any alterations in phenotype (Müller *et al.*, 2007). Harding *et al*. (Harding *et al.*, 2010) also observed detectable AFLP fragment differences between pre-cryopreservation *Euglena gracilis* cultures and samples after exposure to cryoprotective treatments, with alginate encapsulation and liquid nitrogen, mostly related to the appearance of new fragments on the AFLP patterns. However, they considered that this may have been due to DNA methylation rather than genetic changes.

The results obtained in this work provide further evidence of the genetic and phenotypic stability of cryopreserved samples of green microalgae. Both protein and AFLP analysis provided similar profiles when comparing cryopreserved and non-cryopreserved samples. However, there is still room for more detailed analysis on possible consequences of freezing and thawing protocols on cultures, particularly for the production of desirable secondary metabolites. In addition studies employing non-optimized cryopreservation protocols, or taxa with greater cryo-sensitivity would allow the improvement in understanding, both on the effects on algae and potentially how methods could be optimised to avoid such injuries.

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6 Management and Quality Control in Cryopreserved Biobanks

6.1 Introduction

As outlined in Chapter 1, Biological Resource Centers (BRCs) play an important role on the conservation of biological resources and, subsequently, their provision for scientific research, either for academic or industrial purposes, as well as the documentation and diffusion of associated data (Daniel and Prasad, 2010). They act on the interface between users and providers which usually do not possess the expertise to maintain the biological material. Therefore, among their primary functions are actions for the conservation of biodiversity and the development of research in life sciences, acting as repositories of certified reference material and safe depositories for protection of intellectual property and providing the expertise for the formulation of government policies on the conservation of biological resources (OECD, 2007).

The BRC is also responsible for maintaining a comprehensible information database on its holdings, as well as means to supply documented and authenticated cultures for the most diverse applications (Benson, 2008). Thus, these centers become essential aspects for the further development of research, providing the basis for the reproducibility of experimental conditions at different locations, offering holdings which should conform to the required standards of quality of samples. Therefore, the development and adoption of controlled protocols of maintenance are a requirement for the correct management and quality control of cultured strains, guaranteeing their viability, genetic stability, purity and authenticity on strains held for a long time (Benson, 2008).

The authenticity and quality of the strains are keys to the value of any cryopreserved material (Smith and Ryan, 2008). Thus, steps should be incorporated on the protocol to ensure the correct identification and classification of the strains maintained at the BRCs. This will help with the traceability of samples, facilitating the exchange of research information and expanding the informational database available. It can also lead more directly to the adoption of the adequate approaches for culture cultivation and preservation, improving the quality of the service provided by the culture collection. Assuming adequate precautions are employed to prevent mislabeling, or mis-transfers, the taxonomic identification does not need to be undertaken at a regular basis (Smith and Ryan, 2012). However, basic training of the laboratory personnel to perform regular checks on the organisms could also be a useful tool for the quality control of the material available.

The adoption of a best practice protocol for the optimization of the services of safe keeping and deposit of samples in the BRCs, as well as procedures for quality control checks are pre-requisites for the establishment and maintenance of BRCs, supporting the basis of the services offered by each organization providing cultures for use by the scientific community. Quality assurance is the key tool to ensuring fit for purpose materials, focusing on the authenticity of the biological materials held, as well as the assurance of long-term stability and quality control of cell cultures, cell lines and genetic material (Harding, 2004).

The OECD Best Practice Guidelines (OECD, 2001) offers a framework for the steps associated with obtaining and providing genetic materials, namely the acquisition, maintenance and provision of biological materials and, therefore, on the management of BRCs. In this chapter, the application of these guidelines, on three different functions: receipt, preservation and services provision, will be discussed for the development of individual protocols, which will ensure that strains retain key features and are consistent when originated from different suppliers, which will offer a reliable basis for research and development in different laboratories. The work detailed in this chapter, theoretical and practical, has been "steered" by the experiences gained on working at the Culture Collection of Algae and Protozoa (CCAP), Oban during a Research Internship, under the FAPESP scholarship "Bolsa de Estágio de Pesquisa no Exterior - BEPE".

6.2 Sample Acquisition

BRCs should include in their documented policies the restrictions on the material accepted at their catalogues, since some organisms might require specific containment services to avoid harm to staff or other stock cultures. As long as they conform to the remit of the collection in question, cultures to be incorporated may originate from different sources, which includes the isolation of new strains from environmental samples and the deposit from external sources for safe-keeping during research, or where the BRC is registered as an International Depository Authority (IDA) for patent processes under the terms of the Budapest treaty (Budapest Treaty Regulations, 1977; Benson, 2008). However, to ensure the continuity of the quality of services provided, independently of the source of the material, its accession into the culture collection inventory must follow a controlled protocol i.e. an appropriately detailed Standard operation procedure (SoP) to avoid the subsequent dissemination of incorrect information or even contamination of other strains during the maintenance period. This protocol should include steps of identification when necessary, or at least

confirmation of identity of samples to be incorporated on the maintenance regime and also the detailed process of allocating an accession number which will identify the strains on the collection database going forward (Janssens *et al.*, 2010).

6.2.1 Isolation of new strains from environmental samples

The isolation of strains from fresh environmental samples is an essential source of new and potentially economically interesting material for research, opening a new range of opportunities for the development of products (Spolaore *et al.*, 2006). There are many different techniques applied for the isolation of microalgae cells, and independently of the protocol applied, fixed steps should be carefully followed to avoid contamination or loss of samples (Andersen and Kawachi, 2005). When isolating new material from environmental samples, it should insured that cultures are always originated from a single cell/filament/colony, avoiding the possibility of different clones from the same species in a culture, which could cause problems for genetic and physiological analysis of the strains.

Before the full incorporation of a new isolate into the maintenance regime of the culture collection, it should be successfully cultivated and tests must include studies on how to optimize its maintenance protocols, including identification of the organisms. These can be performed directly in the facility when staff has the adequate training for the task, or could be a service requested externally, when more expertise on the subject is necessary. Although the identification of microalgae has traditionally been approached by morphological characters, some closely related groups require more refined analysis for their distinction, raising the need for more modern methods of taxonomy, as the application of molecular techniques, including DNA barcoding (Bellinger and Sigee, 2015).

When dealing with unknown material, the origin of the water sample should be considered and the identification process should be carefully handled to avoid the mistaken acquisition and manipulation of dangerous material and the contamination with undesired organisms, which could have disastrous effects for other stock-cultures (Andersen and Kawachi, 2005). Experience at the CCAP has demonstrated that there are significant risks of accessing fungally contaminated cultures, or materials that could be infected by mites or other metazoans (Day, pers comm).

All information available on the origin of the material should be fully recorded, including physical, chemical and other environmental characteristics at the time of collection. This step may be a determinant point in the definition of maintenance regimes and the culture media better suited for the growth of different groups of organisms. It could also be of interest for future biogeography studies. Furthermore, the traceability of an organism is important on the definitions of the Convention on Biological Diversity (CBD, 1992), which instates the authority of States to determine access to genetic resources under their jurisdiction as part of their sovereign rights (Buck and Hamilton, 2011) and also for compliance to the guidelines for the access, benefit sharing and utilization of genetic resources defined by the development and signature of the Nagoya Protocol (Kamau *et al.*, 2010).

6.2.2 Deposition of samples

The maintenance of samples deposited by outside providers is among the delimited functions of BRCs. These samples may either be directly incorporated into the collection catalogue and thus become publically available to the user community, or be maintained only for a determined period of time as a provided service for their safe-keeping during research or patent processes, with the BRC functioning as a international depository authority. This service is mainly focused on organizations which do not possess the facilities and expertise for maintaining their samples during the commercial or academic exploitation of organisms. The deposition of samples in recognized BRCs might also be a requirement in publication or patent processes, which requires that organisms must be available for the validation of results (Sekar and Kandavel, 2004).

In all BRCs the accession of deposits must be documented and risk assessments should be implemented for the receipt and storage of the material (OECD, 2007), and incoming samples should be handled in suitable local facilities for manipulation of biological material. The latter is key to ensuring safety of personnel and whilst algae are considered to be Class I organisms i.e non-hazardous to man, animals or agricultural plants (Wilson and Chosewood, 2007) and thus they present no risk to man; however, some contain potent toxins (Van Dolah, 2000; Codd *et al.*, 2005) and there is at least one described pathogenic alga (*Prototheca*) capable of infecting man and other animals (Jánosi *et al.*, 2001; Lass-Flörl and Mayr, 2007).

During the process of sample deposition, the provider must also inform all available information on the culture, e. g. name of organism and identifier, the depositor's name and address,

the origin of the strain with assurance that materials fulfill the requirements of the CBD (United Nations, 1992) with documented material transfer agreements, date of isolation and personnel responsible for isolation, the geographical origin of the material, any previous accession numbers in different collections, growth media and conditions and possible hazard information. However, the receiving collection is still responsible of performing authentication, purity and viability tests on cultures, including the formulation of a maintenance plan with periodic controls to guarantee the survival of samples.

6.2.3 Taxonomic identification of samples

Although BRCs are not obligatorily required to employ taxonomy expertise, quality controls to confirm the sample purity, identity and viability should be performed upon receipt (OECD, 2007). This step can also facilitate the development and optimization of the maintenance regime of samples. It is also recommended that the collection perform continuous checks of the organisms maintained, to avoid errors, from mislabeling of samples to cross-contamination between cultures. Cultures maintained in liquid nitrogen after long periods of time must also be validated with taxonomic evaluation, generally on the basis of gross phenotypic characters, after thawing, before distribution or application in research projects (Smith and Ryan; Smith and Ryan, 2012). Thus, at least some basic training of the staff on taxonomic techniques could be interesting for the routine quality controls of samples.

Also, the employment of genetic techniques for the identification of samples, including the development of genetic barcode protocols, is commonly a requirement, or at least highly encouraged by many scientific publishers as criteria for acceptance of papers (Tautz *et al.*, 2003; Sekar and Kandavel, 2004). Therefore, the training of personnel on the application of molecular techniques and interpretation of the data generated can be considered to be a determinant aspect on the quality of the service provided by the BRC.

6.2.4 Axenicity of strains

Whilst in the context of most BRCs strain axenicity is a pre-requisite, for algae this is often not the case and uni-algal cultures are the norm. However, some further applications might require the axenicity of samples to progress the studies. The “cleaning” of cultures might be a service offered

in the culture collection. It must be recognized, that some cultures are intrinsically connected with other organisms, and dependent on them to survive (Droop and Elson, 1966), and the elimination of the bacterial consortia could lead to the death of the microalgal strain, for example many algae are auxotrophic for vitamins (Croft *et al.*, 2005) and thus require either the presence of commensal bacteria, or appropriate additional vitamins incorporated into the medium.

6.2.5 Accession number

Each organism added to a culture collection will receive a unique collection number which should be maintained as long as this strain is kept in this collection, and will never be reassigned, even if the strain is later discarded, or lost.

The process of allocation and the numbering format can be determined by each culture collection to better suit its needs. At the Culture Collection of Algae and Protozoa (CCAP, UK), the cultures are numerically categorized according to their genera and species/strain, receiving an accession number based on this classification with the first number indicating the genus and the second the strain e.g. *Chlorella vulgaris* 211/11b, where 211 = *Chlorella* and 11b is the strain designation (note in many cases multiple strains of the same species are held in BRCs). This system offers an easy analysis and identification of organisms, simplifying the overall organization and management routine for the culture banks. However, this method has also a recognized major disadvantage, particularly when working with microalgae where taxonomic changes have regularly occurred over time. As determined by the best practices, once an accession number is designed for a strain, it cannot be changed. As a result, considering the accession number process in CCAP, there are numerous examples of samples where the same genus may be listed under different numbers e.g. the marine Prasinophyte genus *Tetraselmis* is listed under both 8/ and 66/. This approach also has a limitation on the number of genera and strains which can be allocated in each group, and, as more and more genera have been revealed by improved taxonomy techniques, they can surpass the capability of the numeric system, turning this into an organizational problem, with the need to increase the genera designation to four rather than three numbers. Other collections have employed different approaches, e.g. the SAG collection in Germany where new strains are listed sequentially on the basis of the date of accession e.g. SAG 86.105 would be the one hundred and fifth algal strain accessed into the SAG collection in 1986. Each numbering format has their positive and negative points, and for each collection one method can be chosen to better suit their needs. In general, to

avoid limitations on the number of strain, a sequential numbering would be appropriate, making the possibilities limitless. However, the organization and maintaining the cultures listed using this approach may be problematic, as different groups of microalgae have specific requirements for sub-culturing, and a sequential numbering system does not provide the possibility of a quick Q/C check to assess if strains have been inadvertently mixed/ placed in the wrong location in an inventory or incubator.

A further consideration about allocating accession number is the possible option of a temporary or provisional number for use during the deposition process, which would only be for internal use within the BRC, before actual inclusion on the culture catalogue and, therefore, the public access. This procedure can avoid the designation of accession numbers to non-viable cultures, but also requires very careful processing by the collection staff to reduce mixing or mislabeling of samples, or even the loss of not correctly preserved samples, preventing the requirement of traceability of strains.

6.3 Management of cryopreserved BRCs: Sample maintenance and database control

Cryopreservation and freeze-drying are, currently, the most widely applied techniques for the preservation of biological material (Day and Stacey, 2007). The maintenance of samples in an arrested state after cryopreservation is theoretically able to upkeep their viability and stability for long periods of time, potentially for decades, many hundreds, or even thousands of years (Grout *et al.*, 1990). Furthermore, this approach has the potential to avoid loss of characteristics, including morphological and functional changes that have been previously reported for both cyanobacteria and eukaryotic microalgae maintained by serial transfer (Day and Fleck, 2015). For as long as the conditions are “favorable” i.e. thermal stability is maintained at a temperature well below the eutectic temperature and all cells remain vitrified, the material cryopreserved could be maintained for undetermined periods of time (see Chapter 4). However, as the process is very stressful for the organisms (Day and Fleck, 2015), any variations during the protocols, before, during, or after cryopreservation can easily cause irreversible cell damage, leading to the loss of samples (Day and Brand, 2005). This lack of “robustness” of the process indicates the need of the adoption of very controlled protocols for the preservation of samples and also, for quality control of stocks over time.

The correct management and quality control of the maintained samples as well as the development of an updated database are crucial aspects for the establishment and success of a

cryopreserved biobank of microorganisms (Benson, 2008). Organisms in an arrested state, when immersed in liquid nitrogen, may be maintained for unlimited time while under appropriate care so that the strain viability is maintained, as already discussed in Chapter 4. However, while the viability of samples is maintained by the ultra-low temperatures, the informational database must also be correctly managed to provide the required traceability of samples to their original strains, as all as permit quality controls on the authenticity of samples after storage, which should guarantee that viable samples maintain their due value according with linked published material.

Many factors can affect the samples management when samples are stored for long-term maintenance. The correct labeling of samples is an example of possible source of errors and loss of cryopreserved strains. Also, the correct position of samples in an organized database is essential, providing information for an easier and faster reach of samples, which can be a differential aspect on the maintenance of viability (Day *et al.*, 1997), and as a possible source of corrected information for samples with mislabeling problems. All these points are definite aspects for the validation and tracing of cultures maintained in a biobank, which is a requirement of a BRC.

As part of the process of developing a “fit for purpose” quality management system for the Coleção de Culturas de Microalgas de Água-Doce – UFSCar (CCMA-UFSCar), a case study was undertaken on the CCAP Cryostorage management system. The cryobank at the Culture Collection of Algae and Protozoa (CCAP – UK) has maintained samples for over 40 years in liquid nitrogen storage. During this time many samples have been transferred between different cryostat/refrigerators and inventory systems, as the life-expectancy of a vacuum dewar (the standard refrigerator format used for liquid nitrogen storage) is 8-20 years.

As might be expected for a historical collection, “casualties” can occur in the maintenance of samples and errors in database updating. Over the lifetime of the cryostorage facility systems have evolved from card indices, to hard-copy books, to spreadsheets and digital databases. Furthermore, methods and protocols have developed over the years as has the equipment and material available. These issues have the potential to affect the viability and validation of conserved samples, as there is the possibility that over the storage period there may have been a re-warming of the samples, or labels might be lost and samples misplaced, complicating the tracking of samples to their original strain number.

The above concerns provided a stimulus for a comprehensive “stock-taking” exercise. Thus, a study was developed at the CCAP collection to investigate the accuracy of the current database and

storage, confronting the available information on the digital updated database with the samples stored and maintained at the collection cryostore. The aim was to utilize the information obtained to further develop cryopreservation protocols and quality controls, to reduce the number of errors and, to verify the quality of the product stored by this system. Also, as part of this exercise a barcoding trial was undertaken with the objective of testing its practicality and potential to reduce errors associated with poor documentation, or transcriptional errors between the banding of new materials and database updates.

6.3.1 *Material and Methods*

6.3.1.1 The CCAP Cryostore

Over the past >40 years the cryopreserved samples held at CCAP have been housed in different cryostats/refrigerators/dewars ranging from those capable of holding from 1,000 to 30,000 2 ml vials (The standard format used to cryopreserve algae at CCAP). The current cryostore, maintained at the Scottish Association for Marine Sciences (Oban, UK), consists of four main storage (*Working*) cryostats, each capable of maintaining up to 6000 samples, and a Backup/Master cryostat, with the same specifications as the other storage dewars, where a reduced number of samples from each cryopreserved batch are maintained in more controlled conditions (Fig. 6.1). The maintenance of replicate samples in separate locations is recommended to avoid the loss of all current samples in case of unpredictable events which could affect the viability of samples (Benson, 2008). The current protocol applied at CCAP determines that all cultures are cryopreserved in 15 samples batches (Day and Brand, 2005). From these, three cryovials are after overnight storage in liquid nitrogen immediately thawed for the quality control of the process and viability analysis, while the remaining twelve samples are temporarily stored in the *Working* cryostore. The corresponding information is transferred to the database immediately following the storage process. After the regrowth of cultures and viability analysis of samples, if any of the batches is found not viable, the whole batch is discarded. However, if the organisms had successful recovery and growth, two of the remaining samples are transferred to the *Backup/Master* cryostat for the secure storage of material, while the remaining ten samples function as culture repositories for the culture collection and are maintained in the *Working* cryostats. Whenever the stocks from the main cryostats are running low, new batches

are frozen, and the whole process is repeated. All information on manipulation of store cryopreserved samples must be updated at the database software after the process is completed.

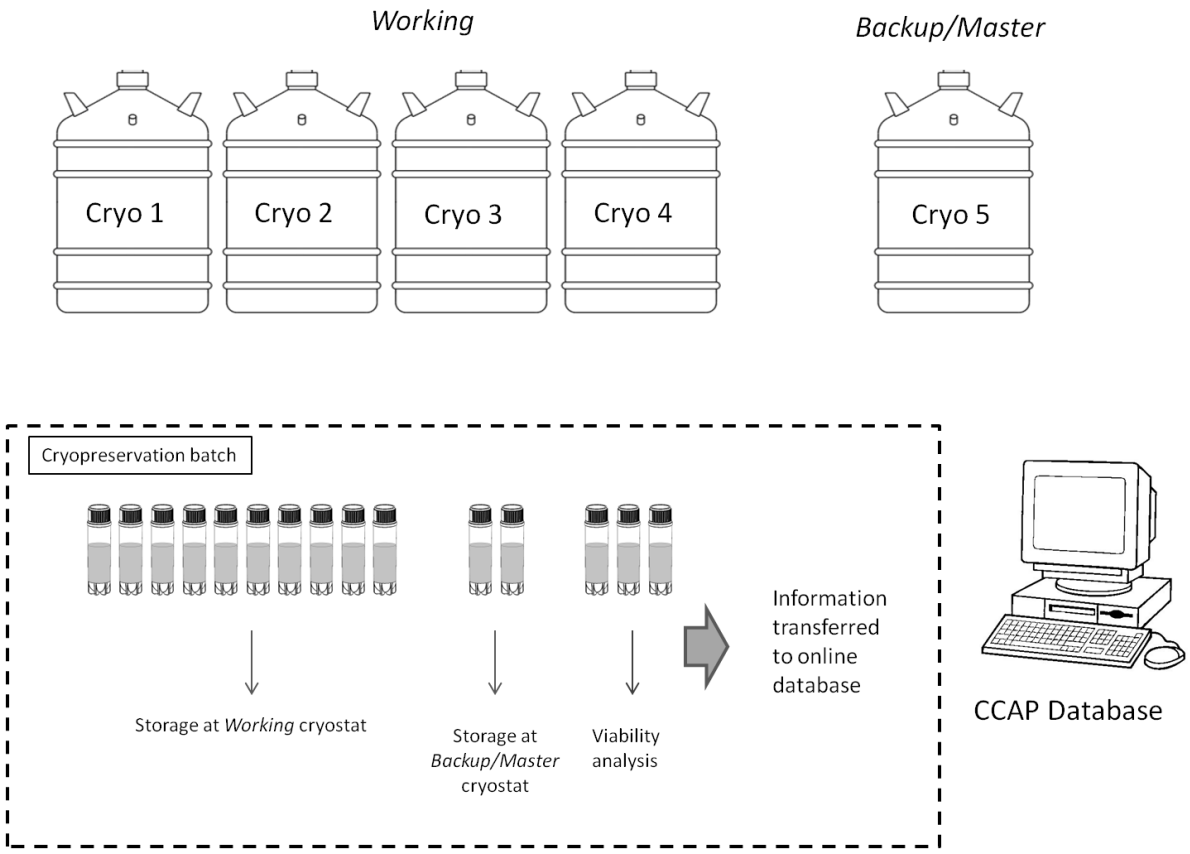

Figure 6.1 Schematic drawing of the CCAP cryostore format, which is composed by 4 main *Working* cryostats and a more secure *Backup/Master* cryostat, and of destination of samples from each batch in a cryopreservation event.

6.3.1.2 CCAP Database

The current CCAP database maintains the information in organized tables which are updated and controlled by internally developed software. The main objective of the system is to map the location of any individual sample in the inventory system (Fig. 6.2). Along with the strain accession number and name and the placement of the vials, complete information about the cryopreservation details, viability and personnel responsible for the process are entered, archiving the whole process for facility of access to information later. However, since this process is a recent development of the

database, the older cultures still present some missing information caused by human error on taking or transferring the information available from the older inventories, or even some details not properly recorded at the time of freezing. Furthermore, for the more recent cryopreservation procedures, this manual system requires careful and efficient transference of relevant data after the freezing process is complete, leaving a few gaps in time where information might be lost or mistakenly entered, as for any changes in the inventory, i.e. the removal of samples for re-growth or quality control need to be immediately updated in the database, or this might result in conflicting information later, as samples expected are not found in the inventory.

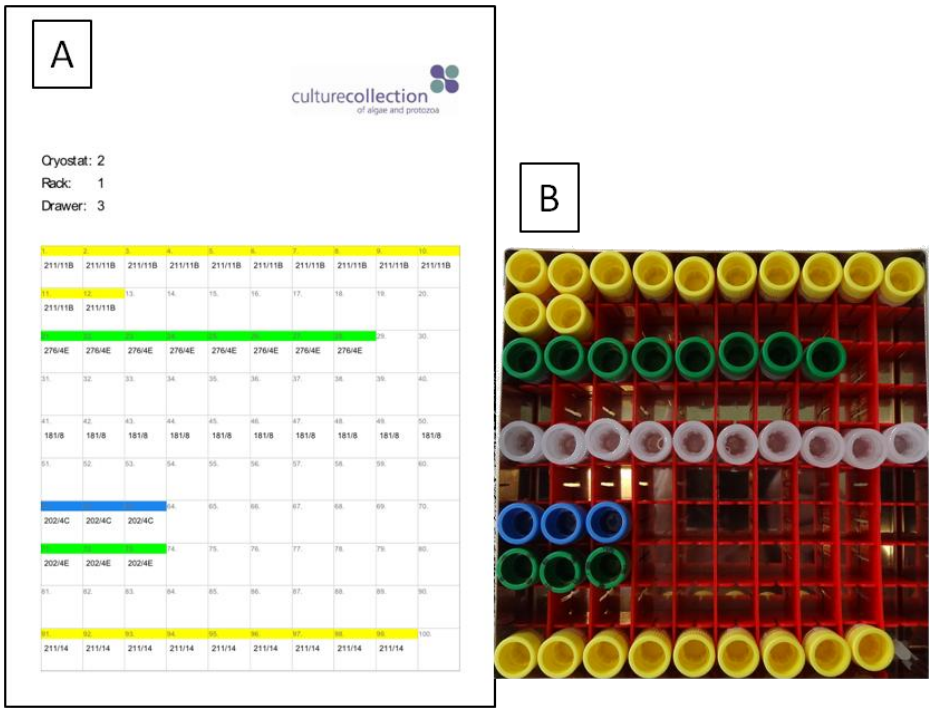

Figure 6.2 Drawer of CCAP Inventory (a) with corresponding map generated by the Cryo database (b)

6.3.1.3 Methodology for database error analysis

In order to analyze the main points of concern for the management of a cryopreserved collection to obtain the expected standards of authenticity and viability defined by the best practices, a comprehensive assessment of the CCAP store was undertaken. This involved noting and categorizing all differences observed when verifying the information available at the most recent database with the real placement of samples on the physical inventory. For this, maps (Fig 6.2 A)

from each drawer of the five storage dewars were generated from the database software, each properly identified and printed for ease of access during the practical exercise taken at the CCAP cryostore. Using the maps and the required safety equipment, i.e. gloves and goggles, the analysis was conducted observing the recommended measures for secure handling of liquid nitrogen. For this study, the information obtained from the database was directly compared with actual material contained in the storage container. Thus, drawers were individually removed from the dewars, and while maintained immersed in LN to avoid variations in temperature, each cryovial label was verified and the information was compared with the data available. Notes were taken on the individual placement of vials, accession number for strains and date of freezing of samples. All differences were noted and summarized in categories for their quantification and easier access and interpretation of results. The categories of errors were detailed in Table 6.1, together with a brief description of the analysis for each group: labeling, position, presence/absence of vials in drawer when compared to database and experimental material stored. The placement of vials and the information on the database was maintained unaltered during the procedures. After complete analysis of each drawer, it was quickly returned to its original position, to avoid generating any more conflicting information which could have damaging consequences for the collection. This process was repeated in all storage dewars, including the *Backup/Master* cryostat.

The presence of experimental material, from different research projects maintained by the CCAP students was considered separately from the main exercise, and the samples identified as “experimental” during the first phase of analysis were not considered during further analysis of the results.

Table 6.1 Errors categories annotated during the cryostorage study of the CCAP cryostore

Errors Main Category	Secondary Category	Description
Missing vials		Samples were listed as available on the database, but corresponding cryovials were not found in the cryostat.
Missing information on the database		Cryovials were present in the cryostore, but no corresponding information was found on the database.
Wrong position		Samples were represented in database in different locations than real position in cryostat.
Label Errors	Completely different label	Labels on samples in the cryostat were different from the information on the database.
	Corrected labels	Labels in cryostat were different than database; however, annotations were available with information on the differences.
	Additional digits	Information on database was missing minor information, with smaller errors. Might lead to confusion of strains from the same species.
	One digit different	Information on database differed from cryostat in one digit. Might lead to mixing of species/strains.
	No label	No information on cryostat samples to confirm information from database. Usually older samples.
	Label unclear	Information on cryovial was unreadable due to fading or illegible writing of numbers.
Mixed Categories	Vials mislabeled and in wrong position, or	Cryovials had labeling issues and were found in the wrong position in the cryostat. Identifiable error, but could cause main problems.
	Vials not in database and unlabeled	No information on database or cryovials on the content of samples.
Drawer errors	Wrong position	The whole drawer was in a different position than found in database, however positioning of samples was right.
	Whole drawer error	All samples in the drawer had position errors. Errors were accounted individually in each category.
Experimental Samples		Samples with different labeling pattern, or those that should have been discarded after an experimental period. These were annotated, but not accounted as errors in this study.

6.3.1.4 Methodology for barcoding trial

For the success and maintenance of quality of the services provided by BRCs, the quality of samples is as important as the informational database associated with samples. Since the current database software requires the manual addition of the data from a cryopreservation event after the whole physical process is completed, the decoupling between the cryopreservation protocol and the update of digital database may be one of the main sources of errors observed for the management of cryopreserved stores. One possible alternative to reduce this source of errors might be the application of a barcoding system, which will require a more direct link between both processes. Although there are many barcoding systems available intended for application on the easier management and access of information for cryopreserved material, they are still under-utilized due to restrictions by the cost of material and ease of use on ongoing storage management systems. Therefore, the practicality and potential of employing barcoding systems to improve the traceability and management of the cryopreserved stocks of conserved materials was tested at CCAP using a new barcoding system (FluidX, Cheshire, UK) and the corresponding protocol for storage of samples was developed and tested for application with the CCAP database (Fig. 6.3). Classic protocols for cryopreservation of microalgae were compared with the developed protocol to verify the applicability of the process and the possibility to incorporate the different steps on the ongoing database for the culture collection. The equipment tested included the 2D barcoded cryotubes (FluidX) and the FluidX Scope^{TM} USB Single Tube Reader (FluidX, UK). Also, alterations were made to a test version of the CCAP cryo-database information software to include the new information generated from the barcoding systems. Tests were performed with the new protocol to verify the practicability of the process when adapted to the CCAP laboratory.

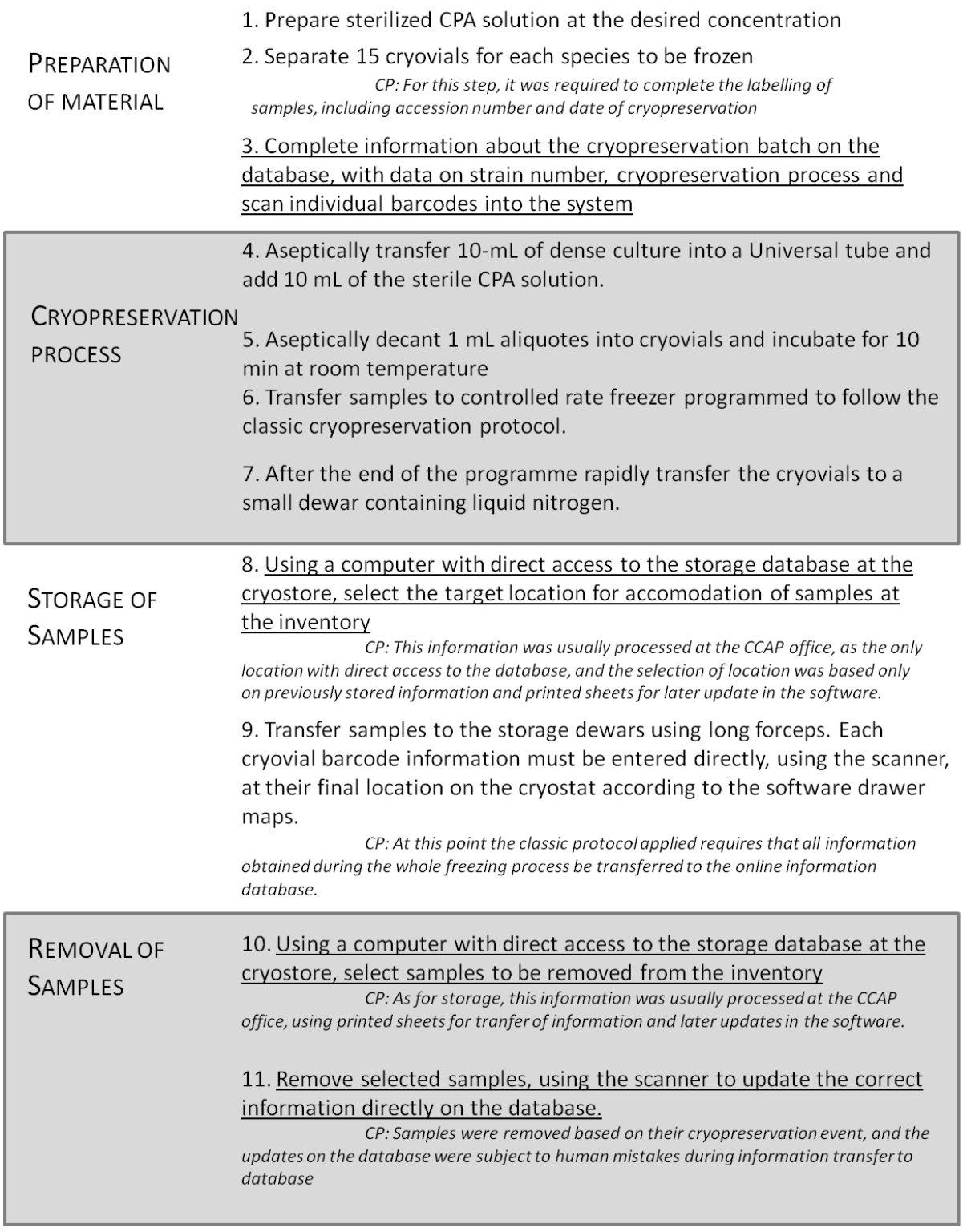

Figure 6.3 Protocol for cryopreservation of samples with the new barcoding system highlighting the points where adaptations were needed from the classic maintenance protocol.

6.3.2 *Results*

6.3.2.1 Database error analysis

The results on the exercise of qualifying and quantifying the errors found on the CCAP cryostorage system were classified and summarized in the Figure 6.4, which shows the percentage of errors in relation to the total number of samples available in each cryostat.

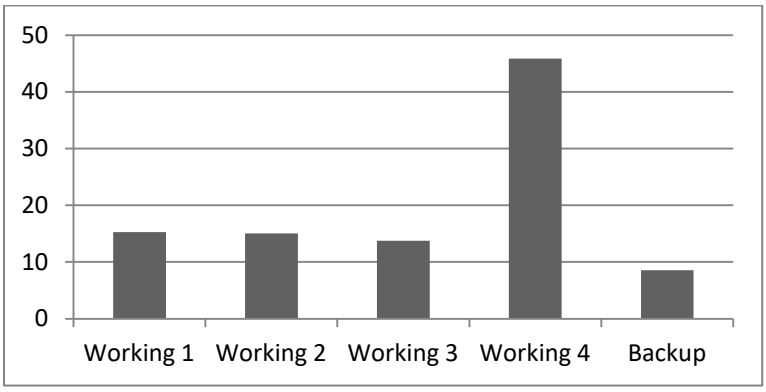

Figure 6.4 Summary of the errors with the number of errors as a percentage of the total number of vials on the cryostats

The higher percentage of errors was found on the *Working* cryostat number four, which is comprised mostly of marine samples, and is the oldest dewar currently being used for storage at the CCAP cryostore. It also held the highest number of experimental samples, which were stored for ongoing CCAP projects. The newest cryostats *Working* 1, *Working* 2 and *Working* 3 had similar percentage of errors (around 15%), and these were probably related to more constant manipulation of the samples stored in these cryostats, and the higher demand for samples in the routine collection management. The smallest percentage of errors was found on the *Backup/Master* cryostat, where viable samples are transferred as a security measure for the maintenance of cryopreserved samples and which requires much less constant manipulation and removal of samples.

A more comprehensive summary of the percentage of errors, for each category annotated, has been outlined in Figure 6.5.

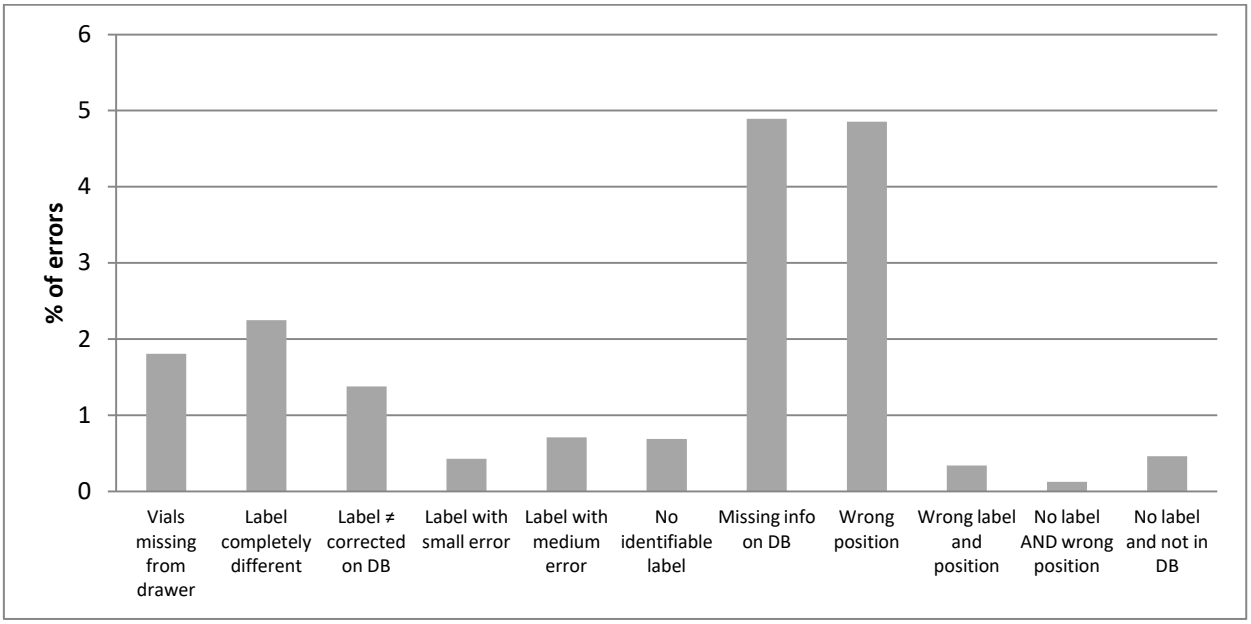

Figure 6.5 Summary of the frequency of errors for the whole cryostorage system at CCAP considering the total number of cryovials stored at the CCAP collection.

The main group of errors observed in the stock-taking quality-control exercise was related to the transfer of information from the cryostat to the database, as many samples were found in the "wrong" position in the physical dewar, when compared to their recorded location on the database. In general, these samples were close to their expected location, these errors were probably related to difficulties on the removal of a specific sample, which was exchanged for one of the same strain from the same batch found directly next to it. Another major contributor to the percentages of errors were that whole batches of samples that were loaded in the cryostats without the corresponding information being logged on the database. These were easily identifiable as the number of vials misplaced usually ranged from 10-15 and these samples generally had clear identification on vials, including the recent date of freezing, which allowed them to be traced to the operative who had made the mistake of not logging the data, without much loss of information.

However, these simple errors were not always the case, as some samples were found without any identification on vials to confirm the information available in the database, and in a few cases, the information available in the vials was completely different from the information on the database. These are more complex errors which could lead even to the loss of samples, as they lost their traceability to their original strain and therefore, all parameters for quality control of authenticity and properties of samples.

A more detailed categorization of the errors observed in each cryostat group is presented in Figure 6.6.

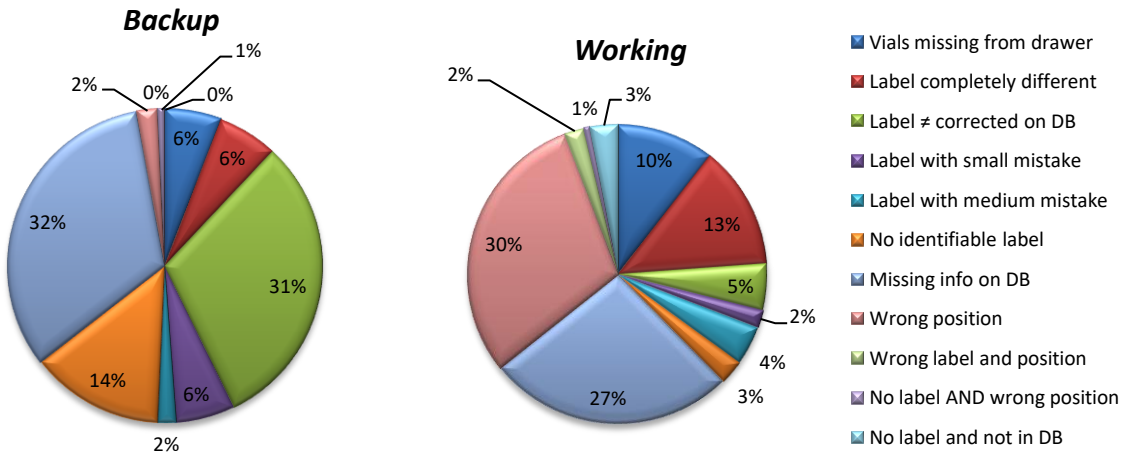

Figure 6.6 Frequency of error categories in each cryostat as a percentage of the total errors in each cryostat.

The advised usage of an individual cryostat is 7 years (Day, personal communication) therefore many of the samples in cryostore at CCAP have had "an eventful history" with several transfers between cryostats during its 40 years of storage of samples in liquid nitrogen. This is reflected in each of the cryostats organization and information control. For the main *Working* cryostats, one major source of errors (reaching 30% of the total found) was the positioning of samples, which was found different from the information stored on the database. These, together with the number of samples found to be missing from the drawer while their information and presence were still accounted in the database (10% of the total errors found) accounted for the most errors found in these containers. These could be consequences of the more constant handling of the samples at these containers, as the most stable *Backup/Master* cryostat had smaller amounts of these errors, which together, corresponded to only 8% of the total number of errors found for this cryostat.

For the *Backup/Master* cryostat, the main sources of errors found were related to changes during time on the strains information recorded on the database after the freezing process; however, these changes were already annotated (31%), lessening their lasting effects for the management of these samples. In the main these were due to taxonomic changes/ confirmation of cultures identity using newer molecular techniques over their years of storage. For the *Working* cryostats, a reduced percentage of the errors were found to reflect these errors (5%).

Overall, for both *Working* and *Backup/Master* cryostats, a major source of errors for the management of cryopreserved samples (reaching 27% and 32%, respectively) was the failure on the process of updating the information on the database after the cryopreservation process is complete This resulted in many samples being stored at the containers without their corresponding information being entered on the database software, or at least missing their final placement on storage, which only indicates blanks on the drawer maps were those samples are currently stored.

6.3.2.2 Barcoding trial

The new protocol adapted had main changes in two steps of the cryopreservation process:
I) Prior to the cryopreservation of samples, all the information was added at the database, completing the data on the strains to be frozen and also cataloguing all the cryovials separated for use with each strain to be stored;
II) After the protocol, when samples were transferred to the storage cryostats, a computer with database access was needed in the cryostore, as each vial was included in the database and its position recorded individually, based on its barcode.
These differences in the protocol facilitate the process of transferring the information from the physical inventory to the database software, reducing the opportunities of errors in placement of samples during the storage and removal of the material.

Although changes were incorporated on the protocol, the user practicality of the process remained the same. It is necessary to highlight that adaptations were necessary to the software database to include the receipt and storage of barcode information from each vial into their correct batches of cryopreserved material. As anticipated, the barcoding system from *FluidX* tested was efficient for processing the alterations proposed for the new protocol, justifying the applicability of the system for cryopreserved sample storage at CCAP.

6.3.3 Discussion

The overarching aim of this chapter was not only to explore how and when errors can occur during the process and management of cryopreserved materials, but to look at this holistically in the context of enhancing quality of the materials held and thus, the services provided by an algal BRC. In the below sections the issues associated with the quality control exercise undertaken on the CCAP cryostore, the possible use of barcoding to improve the management of cryopreserved samples and other aspects of quality management are discussed. The aim was to develop best practice for future additions to and management of the cryopreserved stocks of algae held in CCAP and the possible application of these methods in the ongoing development of the cryopreserved biobank at the Coleção de Culturas de Microalgas de Água-doce (CCMA-UFSCar).

6.3.3.1 Error analysis –implications to improving Quality Control

Although the viability of samples successfully cryopreserved should be maintained while the liquid nitrogen levels are secure, as discussed in Chapter 4, the control of the storage system and the correct management of samples are crucial for the maintenance of samples value and the reliability of the material (Benson, 2008). The success of the storage system is dependent not only on the quality and reliability of the storage vessels, but also on the accurate documentation of the storage inventories (Day and Stacey, 2007). Thus, the error analysis, as performed with the CCAP database, brought to light a few protocol and quality control problems found during long term storage of microalgae samples, which could affect the traceability and authenticity checks of samples. Over the >40 year period of storage of cryopreserved protists at CCAP, the methodologies for cryopreservation varied. Changes were in part as result of changes in the researcher responsible for performing the process, methodological improvements and the available equipment at the time. These issues had direct responses on the quality of the material and the amount of information stored for each cryopreserved batch, as well as the maintenance routine for the whole frozen culture collection. Thus, one of the larger amount of errors found during the assessment of the cryopreserved store, although not completely accounted for or detailed, was the absence of dating on the labels of a large fraction of the samples stored. This was particularly frequent in materials conserved prior to the CCAP relocation from Cambridge (Southern England) in 1986 to Windermere (Northern England) and subsequent relocation to SAMS in 2004 (Day *et al.*, 2004). These older samples were most comprehensively logged on a card index still available at the CCAP archives.

However, throughout the years, a variety of methods were employed to document the procedures, which included individual scientists' lab-books (most of these have not been archived and so much data has been irretrievably lost). These gaps in information might affect the traceability of samples and also lead to the overlook of important information gathered on the storage period, such as temperature anomalies, or unexpected events. Not least, because of the number of people involved over the years, the information available on the cryovials label was not standardized, causing some confusion on the dating and tracing of samples to their original strain data.

The viability, purity, authenticity and stability of samples are key features to be assessed on quality control checks (Benson, 2008). All of these lead back to the necessity of a properly documented database of cryopreservation of the material maintained at the collection stock. This documentation will permit the traceability of cryopreserved samples to their original strain providing the basis for tests of authenticity and properties of samples. It will also allow quality checks on the maintenance of viability of strains during long term storage.

While analyzing the CCAP cryostore, the most serious errors observed were the storage of cryovials without any identification label and/or with completely different information between the samples stored and the information available on the database. This inconsistency in the information could lead samples to either disposed, or requiring to be submitted to a new identification and accession number process, as their authenticity cannot be guaranteed. These samples cannot be traced back to their original strain, creating vulnerability on their quality control. However, this was found only in around 3% i.e. a total of 426 cryovials, which is a relatively small number considering the total number of samples (14496 cryovials) and internal movement events between main cryostats and the period of storage. Furthermore, a significant number of these vials have labelling information, normally the strain number, which has been written on the vial using a red hot wire (Day pers comm). Many of the oldest samples dating from as early as the mid 1970's, which do not appear to have writing on the cryovial have been labelled using this technique. Unfortunately, this method only allows the contents to be identified/ strain number to be verified when the vial is empty and held up to the light. This procedure was discontinued in the 1980's as it became obvious that it limited traceability and sample management. Some minor labeling problems were also found on a larger amount of samples. However, these were less problematic errors, which should not compromise the traceability of samples, considering the amount of additional information available in the database for correct tracking of samples.

The current protocol for transferring information to the digital database at CCAP is dependent on the correct update and insertion of data after the whole freezing or thawing process has been completed. This disconnection between the actual removal, or addition of samples in the cryostat and the update of informational database may be a potentially “problematic” source of problems, particularly for being the probable cause of most of the errors found on the positioning of vials in the drawers, which accounted for roughly 5% (704 vials) of the total number the vials available at the cryostore. The process of removal of samples, is not always a direct process as samples might be stuck due to the formation of ice around the tube and a similar nearby cryovial, with replicate samples can be removed more easily instead. If the information is not correctly transferred to the database after this procedure, it might lead to apparently minor errors on the database. Also, if information is logged onto the database before the vials are physically loaded into the cryostat, some might be placed in the wrong place, resulting in a larger error account (i.e. whole sequences of vials at the wrong place). It is clear that this has historically been common practice and thus is easily prevented in the future by adhering to a more rigorous SoP.

The presence of experimental vials in the cryostat was a large source of database errors, although these were not accounted for in this study for the main comparison between cryostats. These vials have routinely been included, or removed from the inventory drawers, and their labeling and information is quite limited. Ideally, in a culture collection with partial focus on research, an experimental cryostat should be set up, differentiating these samples from the rest and avoiding further errors in their main stock-culture holdings. When that is not possible, experimental samples should be clearly labeled as such, so as a differential these vials from the rest of the banked samples, to avoid unnecessary handling, which could be a source of contamination and viability reduction. As soon as the experiments are completed these drawers should be cleaned and any residual material should be correctly disposed of, or archived for future assessment(s).

This exercise on quality control of the management system highlights, while discussing the best practices of a cryopreserved biobank, the importance of connecting the cryopreservation process directly to its correct update on the related database. Steps should be included, when designing the SoP, to assure that all available information on a stored strain, joint with determinant information on the freezing process, are correctly archived. One possibility to facilitate the link between the cryopreservation process and information processing is the use of barcoding systems for storage.

6.3.3.2 Barcoding systems for cryostorage: providing a solution for human errors?

Barcoding systems can facilitate the correct transference of information between the process of freezing, happening at the laboratory, and the online database. Applying this system, each cryopreserved sample will receive a unique barcode fixed to their cryovial. Thus, during the cryopreservation process it was required, as observed in our tests, that the information be transferred simultaneously to the process, reducing the possibilities of most of the errors observed appearing, which were due to the decoupling of these processes.

This new process also includes changes on the removal of cryovials, as samples can have their barcode read for their removal from stock, avoiding errors on the positioning of vials caused by unexpected events, or human error. No differences were observed on the difficulty and timing of the process, when compared with the conventional cryopreservation protocol that has been applied in the CCAP laboratory, although an adaptation to the management software was required. The inclusion of a barcode system can reduce the possibilities of errors caused by labelling and conflicting information on cryovials. However, it has an added cost for the collection, as its implementation requires the acquisition of dedicated equipment and the offer of database access points at both the cryostat and the laboratory. Also, the cost of the barcoded vials is greater than the commonly applied cryovial, which requires planning and further continuous investment by the collection.

Furthermore, for the routine handling of samples, either for re-growth or quality control checks, the barcoding system offers the advantage of reducing the chances of one of the main groups of errors identified in the CCAP cryostore, namely error on the exact localization of samples in the cryostat. As the removal of a sample using their barcode will directly connect with its individual spot on the drawer, it will be correctly removed on the system, while the classical protocol considered all samples from the same batch interchangeable, originating small errors on the location of samples and empty locations in the inventory.

As suggested in the protocol, the new barcoding system could eliminate the need for labelling the cryovials, as all information is entered directly on the system individually related to the unique cryovial barcode. However, should this step be eliminated, the samples maintenance and quality control becomes entirely dependent on the online database which, as any system, is subject to failure. A crash on the barcoding system could have inestimable consequences for the biobank, leading to the loss of most of the material maintained. Thus, although the barcording system was

efficient *per se* for the management of samples, labelling the cryovials must be still considered a security measure to guarantee the traceability of samples to their original strains.

6.3.3.3 Quality control of cultures

Periodic checks of the stored material must be performed to ensure the quality of the samples available. These must include taxonomic identification checks, to verify the authenticity of the strains at long term preservation as well as essays on the properties of the culture. Whilst human errors are inevitable, good procedures will reduce the likelihood of their occurrence and avoid the mixing of cultures or labeling errors. There are remarkably few documented reports on the traceability of the strains used in experimental approaches. Although there are no legal requirements to authenticate specific algal strains for ecotoxicological studies, the application of ISO standards by laboratories are leading to the necessity of quality assurance of bio-products and services (Day *et al.*, 2007). In a study with *Chlorella vulgaris* strains maintained at different culture collections, Müller et al. (2005) revealed the importance of recording the actual strain used in experimental work, since genomic differences were found between strains from the same species. It is almost certain that these would be linked to differences in quality and concentration of exploitable valuable compounds, or other important characteristics. However, no differences were found between samples from the same isolates, even after long time maintenance in different collections under different cultivation regimes, nor after in excess of 20 years storage under LN (Müller et al., 2005).

For the living cultures maintained in axenic conditions, routine tests to check the purity of samples are also necessary to confirm if the quality of the isolate is as specified on any publically accessible website and/or catalogue for direct application on research projects.

Morphological analysis of samples, using optical microscopy, might not be conclusive for assessing the identity of the samples, as the long-term maintenance of metabolically active cultures might be the cause for selection and loss of morphological characteristics. Thus, after long term storage, it is recommended to apply molecular tools to confirm the identity of samples, as the use of DNA-barcode, for example. However, even if barcoding may reveal that samples have been contaminated, for example with another alga, or mislabeled, they do not provide sufficient data to assess stability or change in expression of the alga's genome, as they are most usually related to only one or two genetic markers (Saunders and McDevit, 2012). For this type of genomic study, techniques with higher resolution are required, mostly assessed using polymerase chain reaction

(PCR) techniques, as the randomly amplified polymorphic DNA (RAPD)-DNA fingerprinting (RAPD) (DeVerno *et al.*, 1999), the Simple Sequence Repeat (SSR) analysis (Harding and Benson, 2000) and the Amplified Fragment Length Polymorphism (AFLP) analysis (Vos *et al.*, 1995; Müller *et al.*, 2005; Müller *et al.*, 2007). Other methods might be applied, for full metabolic profile checks, to verify the stability of strains, including Matrix Assisted Linear Desorption Ionisation Time of Flight (MALDI-TOF), Gas Chromatography and Liquid Chromatography-Mass Spectrometry (GC-MS/LC-MS) (Smith and Ryan, 2012), and these could be more focused on compounds of interest for research or industrial applications. However, irrespectively of the availability of any molecular data, any changes on the morphology of samples along time should be annotated and the records made available for further research on strains, as morphological changes can occur in response to physiological changes.

For the samples maintained in cryopreserved storage, some of the most important continuous checks that need to be performed fall into the category of monitoring and regulating the storage environment temperature. The temperature of storage, as well as the cryopreservation conditions will directly affect the viability of samples (Day *et al.*, 1997). At CCAP this involves maintaining the liquid nitrogen levels in the cryostore, and thus, prolonging the sample viability. However, whenever a sample is thawed for utilization, tests on the viability and identity confirmation of samples should be recorded in the batch database, thus generating valuable data on the maintenance of samples over time. These data would be invaluable for the development of a comprehensive knowledge-base that provides real data allowing substantiation of cryobiological claims on stability, longevity and functionality of cryopreserved materials. Also, identification tests should be determinant on cases of database errors as described in previous sections.

6.3.3.4 Consequences of Quality Control for Services provision

The maintenance of microbial BRCs require, for their continuous function: adequate staffing, technical support, information services and databases and services of culture exchange (Ivshina, 2012). Adequate long-term funding is crucial to support culture collections, for their maintenance and future development (OECD, 2001). However, the financial stability and funding for the culture collections are usually insufficient to maintain and sustain their continuous requirements. As a result, most BRCs usually undertake the provision of services as a method both for servicing stakeholders needs and to generate funding for the support of their activities.

Culture collections have an important role globally in providing the biological resource to underpin research and development; however, not one collection can have representatives of all microorganisms (Ryan *et al.*, 2003). The networking of BRCs is essential for the coordination and cover of broad geographical and political extension. It facilitates the process of enhancing expertise, optimize functionalities and the process to address specific problems that could not be solved individually (Janssens *et al.*, 2010). The exchange of information and best practice between similar facilities is a simple manner to generate a better service to science, while reducing the individual efforts of each group to confront general problems.

Biotechnology companies require stable starter cultures for the commercialization of their products and authenticated reference strains have to be stabilized in cryobanks for patenting purposes (Budapest Treaty, 1977), with collection functioning as international depository authority (IDA) (Sekar and Kandavel, 2004). Also, the supply of known, validated and precisely identified resources is essential for research (OECD, 2007). The applicability of any supplied material on academic and commercial purposes must be properly ensured by the certified BRCs, when those samples management was accompanied by quality checks and validation of the samples. As predicted by the OECD Best Practice Guidelines (OECD, 2007), the BRCs should be accessible to the broad scientific community; however, this access does not need to be free of charge. The ordering and delivery system is usually a large source of income for the maintenance of a functional BRC, to complement the core funding of the facility. At the CCAP, for example, distribution of strains is initiated via an online ordering service, with an automated payment system. Any order requests are usually supplied in a four weeks period, but this time may vary according with specific requirements of the strains requested. The collection also offers the supply of culture media in the form of non-sterile stocks for 5L of media. However, even as a source of income to most culture collection, this ordering system still requires specialized personnel to adequately complete the tasks in sufficient time, which may affect the costs for the funding of the facility.

The training of personnel on the required fundaments on techniques and protocol for identification, maintenance and general organization of BRCs is a requirement for the better approach on the quality of the strains maintained and provided by a BRC. The presence of trained staff members for routine quality checks on the identity of the maintained material helps facilitate and guarantee better results on the authenticity of the material. Also, all conservation techniques applied for microalgae imply the use of aseptically transference techniques to maintain the purity of the samples, avoiding contamination by external groups or even cross-contamination between the cultures held. The personnel training can be an important aspect on networking efforts between

BRCs, which is a requirement for the better development of their own collections and quality control of samples for the further enhancement of science.

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7 General Discussion

Culture Collections are essential instruments for the conservation of algae biodiversity and furthering microalgae research, underpinning the scientific activities of researchers, whether for fundamental or applied research. The commercial and scientific values of these organisms has been increasingly recognized, particularly as the interests on these organisms grow in connection to the development of biotechnologies for the identification and isolation of secondary metabolites, and implementation of their large scale productions for applications on the nutraceutical, nutritional and biodiesel industry (Spolaore *et al.*, 2006; Chetverikova, 2011; Leu and Boussiba, 2014). Culture collections are expected to adapt to these expanding needs, mostly by the expansion on their holdings groups and diversity (Brand *et al.*, 2013). The establishment of cryopreserved biobanks for the maintenance of strains should severely reduce the constant handling of samples which is usual for serial subculture maintenance (Day and Brand, 2005), therefore, facilitating the management of the collection, allowing for the expansion the collection capacity without the substantial increase on the maintenance cost (Brand *et al.*, 2013).

The practical application of these conservation protocols was analyzed for the Coleção de Culturas de Microalgas de Água-doce (CCMA-UFSCar), based at the Phycology Laboratory, Universidade Federal de São Carlos. This is currently the largest microalgae collection in Brazil, and source of samples for a wide range of public and private institutions, subsiding the development of much academic and biotechnological projects. First, the necessary cryobank infrastructure was implemented, and equipment for preparation of samples and freezing of samples at controlled conditions was acquired. Although the establishment of the cryopreserved bank requires an initial investment on specialized equipment; it simultaneously, after successful implementation of the techniques, severely reduces the maintenance costs for cultures, and minimal investment is required for the expansion of the cryobank holdings (Day and Stacey, 2007). As an objective and consequence of the ongoing BIOTA Thematic Project “Biodiversidade de microalgas de água doce: banco de germoplasma e obtenção de marcadores moleculares das espécies

criopreservadas" (Processo FAPESP 2011/50054-4), the CCMA-UFSCar holdings have been almost doubled (from 400 to almost 700 strains) in the last few years, and is now reaching limit capacity for the routine maintenance of actively growing cultures, considering the needs for personnel, designed space and costs of material. Furthermore, recently, there was an observed raise on the reports of external contamination of cultures, and, consequently loss of samples. In the initial approach with a standard two step protocol for cryopreservation, using dimethylsulfoxide (DMSO) as cryoprotective additive (CPA), results were mainly positive, demonstrating the feasibility of this process for the conservation of a wide range of species, in order to reduce the costs for the maintenance of microalgae strains. However, a range of strains held at CCMA-UFSCar are still recalcitrant to the tested methodology, particularly diatoms and desmids and empirical tests must be conducted for establishment of a possible successful protocol for these organisms. Further protocol adaptations were tested for *Chlamydomonas chlorastera*, positive results could be found for the cryopreservation after the change on the applied CPA for cell protection, with the better responses found for the treatment with methanol (MeOH). However, for most of the cultures tested, MeOH and DMSO were interchangeable, with positive results for both the protocols, giving priority to other aspects to be considered for the long term maintenance of frozen microalgae.

The majority of CCMA-UFSCar strains are maintained as unialgal cultures, as they were obtained after the isolation from environmental samples, and most still have an associated bacterial community. For the non-axenic strains tested, the choice of cryoprotectant was found to be crucial for the success of the cryopreservation protocol. Although the results for initial post-thaw viability (% of living cells in the culture) of non-axenic strains had comparable patterns to those obtained for their axenic counterparts, some "contaminated" cultures need further attention during the recovery and development of cultures (Amaral *et al.*, 2013). Cell damage during the process of freezing and thawing of samples, increases the release of organic matter, which can work as substrates for the development of the heterotrophic attached communities (Harding *et al.*, 2010), and, the high proliferation of the contaminant cultures can inhibit the growth of the desired target algal cultures. Furthermore, MeOH is also a possible carbon source for microorganisms,

which could also facilitate the establishment and proliferation of the contaminant cultures (Amaral *et al.*, 2013), directly affecting the population dynamics and the metabolism of the desired microalgae cultures (Grossart and Simon, 2007; Wang *et al.*, 2016).

Although the maintenance of non-axenic strains is usually more desirable for culture collections, reducing the costs with purity checks and facilitating the maintenance conditions, in specific cases, the purification of strains and obtaining an axenic culture becomes a requirement. The purification of microalgae is a time consuming process, particularly when dealing with recent isolates, and one usual approach involves the use of antibiotics (Guillard, 2005). The combinations between the purification process and the conservation of samples using cryopreservation were tested for a few selected strains of microalgae. However, the positive results were limited for one more resistant organisms, while the combination of two potentially injurious processes on more sensitive microalgae led to very limited viability or culture loss.

The studies on the longevity of samples cryopreserved delimited the importance of maintenance of thermal stability for the successful recovery of samples after long periods of storage (Day and Fleck, 2015). The CPA choices were found to be crucial to ensure the maintenance of the viability of samples, particularly on suboptimal conditions of temperature of storage. Although both methanol and DMSO are considered colligative penetrating CPAs, their mechanisms of protection and physical properties including their vitrification point are different (Hubalek, 2003). Therefore, they respond differently to temperature variations associated to the maintenance of samples at temperatures higher than -139^{o}C, conditions in which biochemical and biophysical processes can still occur (Morris, 1981). However, for strains maintained in stable conditions while immersed in liquid nitrogen, the viability of samples was found to extend to over forty years storage, although careful monitoring and regulation of the storage environment temperature, avoiding temperature fluctuations which can lead to the loss of stored samples (Day *et al.*, 1997; Day and Fleck, 2015). To date these are the longest-term quantitative data that have been generated on any cryopreserved materials (bacteria – embryos), although some qualitative information on longevity of bovine spermatozoa and other materials are available (Day and Stacey, 2008 and citations therein).

Different levels of success were obtained for post-thaw viability of some tested organisms, and while the samples maintained in liquid nitrogen have reported stability on the viability rates, the cryopreservation process is potentially damaging to cells, which could lead to genetic modifications and consequently the possibility of the loss of desired characteristics by the maintained strains (Müller *et al.*, 2007; Day and Fleck, 2015). Our studies with green algae indicated the maintenance of culture growth patterns, with growth of comparable robust cultures originated from cryopreserved and serial subcultured inoculum. Further biochemical analysis, with Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-TOF MS), indicated the stability of phenotypical responses between treatments, even for samples exposed to repeated cryopreservation cycles. Similar responses were obtained after genetic analysis of samples, using Amplified Fragment Length Polymorphism (AFLP) analysis, which generated similar fragment size patterns. These results are indicative of genetic and phenotypic stability of the frozen samples (Müller *et al.*, 2007). Analysis of the stability of cryopreserved samples are important in order to validate the cryopreservation process as an usual methodology for the long term conservation of microalgae strains.

Furthermore, the control of the storage system and the correct management of samples are crucial for the maintenance of samples value and the reliability of the material for biotechnological application (Benson, 2008). Any sample provided to final users should be accompanied with a unique identifier of the strain, the accession number, facilitating the technology transfer between research centers and the access to published information on the strain characteristics. When considering a cryopreserved biobank, samples can be stored for decades with very little to no manipulation (Day and Brand, 2005), and many factors can affect the samples management during this period. Thus, the maintenance of the accurate documentation of the storage inventories (Day and Stacey, 2007), allowing the traceability of samples to their original strains is a key factor, ensuring the direct transfer of information between the practical freezing process and database updates, which could be facilitated employing barcoding systems for identification of cryovials.

7.1 Perspectives for future work on cryopreservation at CCMA-UFSCar

Although promising positive results were found for the majority of the tested microalgae, these initial studies are just the ´tip of the iceberg´ as much research and methodological development is still necessary to include all available samples from CCMA-UFSCar to the collection cryobank, particularly the desmids and diatoms, and to further the understanding of the intricacies of the cryopreservation process, while insuring that functional and genetic stability of samples. A few constraint points for the successful achievement and maintenance of viability for strains were further analyzed in this project; however, many points are still to be explored for the successful implementation of this technique for the maintenance of organisms' biodiversity.

Very little information is available on the validation and consistency of cryopreservation methodologies and their effects on the functional and genotypic stability of cultures (Müller *et al.*, 2005; Hédoin *et al.*, 2006; Müller *et al.*, 2007; Hipkin *et al.*, 2014; Day and Fleck, 2015), and most physiological analysis were focused on the production of specific compounds of interest. The employment of the MALDI-TOF mass spectrometry in these initial tests provided a promising overview, although more detailed tests on a wide range of strains is required for the validation and establishment of this technology for analysis of the physiological stability of samples. Tests are currently being expanded to include 18 strains of microalgae and the analysis of the samples submitted to high temperatures stress.

The AFLP analysis for the determination of genetic stability of cryopreserved samples of microalgae was effectively applied for 29 strains of microalgae (Müller *et al.*, 2005; Müller *et al.*, 2007), and variations were found for at least 14 of these strains. In this project these techniques were applied to two different strains submitted to repeated cryopreservation processes; however, the restriction/ligation reactions for *A. fusiformes* produced limited results. Despite considerable efforts to trouble shoot this problem and several adaptations on the protocol, no specific cause was found for this problem. This could be related to complexities on DNA extraction or intricacies on the AFLP technique which will demand further refinement of the protocols before application on a wider range of strains.

While this work has currently been mostly focused on the small green algae (Chlorophyceae, Selenastraceae), which are the major focus of research currently underway in the Phycology Laboratory at UFSCar, there is a vast biotechnological potential on the expansive catalogue of freshwater microalgae available at CCMA-UFSCar, which is still mostly unexplored. The biochemical screening of samples for potential biotechnologically exploitable has the potential to be combined with conservation techniques for validation of protocols on the analysis of stability of samples and their desired characteristics within the microalgae strains.

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8 Conclusions

1. The establishment of a cryopreserved biobank for CCMA-UFSCar was found to be a viable option. Specific research is still necessary to include the majority of the strains currently maintained as living cultures.

2. The standard cryopreservation protocol employed was robust and had reliable levels of viability and capacity to recover for 98% of the small green microalgae strains tested. However, as the complexity and size of organisms was increased, viability levels were severely reduced for this protocol.

3. Cryopreservation of non-axenic strains had no noticeable differences on the viability levels (i.e. the % of living cells) when compared to similar axenic strains. However, the recovery of cultures was severely affected by the proliferation of the heterotrophic associated community, which, in turn, was directly related to CPA choice.

4. Longevity of samples stored at suboptimal conditions of temperature was found to be associated to the CPA employed during cryopreservation protocol, with longer viability for samples maintained using DMSO as CPA, and for the samples stored at temperatures over the eutectic point, loss of viability occurred over a short time period.

5. For samples stored under ideal conditions, i.e. immersed in liquid nitrogen, longevity with no significant reduction in viability levels of around 40 years was demonstrated. However, the correct management of nitrogen supplies is required to avoid episodes of nitrogen depletion which will lead to viability loss.

6. Although the cryopreservation process involves risks of cell damages, which can lead to genetic and phenotypic changes on the preserved material. In general, no significant differences were observed for AFLP profiles or MALDI-TOF MS analysis of the samples tested.

7. The management and control of the cryopreserved collection is essential for the preservation of the strain associated values, providing the basis for traceability and validation of samples and biotechnological techniques, which was corroborated in the assessment of the CCAP cryostore.